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A Solid Lipid Nanoparticle Formulation of 4-(*N*)-Docosahexaenoyl 2′, 2′-Difluorodeoxycytidine, a Compound with Potent, Broad Spectrum Antitumor Activity

Committee:

Zhengrong Cui, Supervisor

Debadyuti Ghosh

Robert O. Williams III

Salomon Stavchansky

Rick A. Finch

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by

Solange Alondra Valdes Curiquen

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Dedication

To Guillermo, Soledad, Denisse, and Florencia my loving family, who always have given me unconditional support throughout my life to reach my personal and professional goals.

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Abstract

A Solid Lipid Nanoparticle Formulation of 4-(*N*)-docosahexaenoyl 2′, 2′-difluorodeoxycytidine, a Compound with Potent, Broad Spectrum Antitumor Activity

Solange Alondra Valdes Curiquen, Ph.D

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Supervisor: Zhengrong Cui

The strategy to improve the lipophilicity of gemcitabine by conjugation with a lipid (e.g. docosahexaenoic acid (DHA) or stearoyl acid) allows to prevent the deamination of gemcitabine by cytidine deaminase, main cause of gemcitabine degradation *in vivo*. In addition, this strategy may confer gemcitabine a strong antitumor activity compared to parental drug. 4-(*N*)-docosahexaenoyl 2´, 2´-difluorodeoxycytidine (DHA-dFdC) is a novel compound with strong *in vitro* and *in vivo* antitumor activity in several cancer models (e.g. pancreatic, breast, leukemia, and lung cancer). However, the toxicity of DHA-dFdC has not been tested in an animal model. In this dissertation, a preclinical short-term toxicity study to evaluate the tolerability of DHA-dFdC was performed in healthy DBA/2 mice. DHA-dFdC showed a dose-dependent toxicity, affecting mainly the spleen. The repeat-dose maximum tolerated dose (RD-MTD) of DHA-dFdC was 50 mg/kg. DHA-dFdC exhibits a strong efficacy at or below its RD-MTD in mouse models of pancreatic cancer or leukemia. Unfortunately, DHA-dFdC has two main issues, chemical instability and poor solubility in water. In this dissertation, a solid lipid nanoparticle (SLN) formulation of

DHA-dFdC was developed to improve its chemical stability and water solubility. The SLN formulation improves the apparent water solubility of DHA-dFdC by 2.6-fold, as compared to a previously developed DHA-dFdC in Tween 80-ethanol-water solution. The SLN formulation as a lyophilized powder also improves the chemical stability of DHA-dFdC. In addition, DHA-dFdC-SLNs showed stronger antitumor activity than DHA-dFdC in Tween-ethanol-water solution in a mouse model when given intravenously. Finally, this dissertation demonstrates in a mouse model that the absolute oral bioavailability of DHA-dFdC in the DHA-dFdC-SLN formulation is 68%. It is concluded that the SLN formulation of the DHA-dFdC (i.e. DHA-dFdC-SLNs) improves the chemical stability of DHA-dFdC and increases its apparent water solubility, and the DHA-dFdC-SLNs can be administered intravenously or orally.

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Chapter 1: Lipophilic Derivate of Gemcitabine as Strategy to Overcome Drug Resistance

1.1 INTRODUCTION

Cancer is a leading problem in public health around the world and it is the second cause of death in the United States (1). There are several options to treat cancer such as surgery, chemotherapy, radiation therapy, hormonal therapy, and targeted therapy. The adequate selection of treatment depends on many variables such as localization, grade, and stage of the disease (2). According to the American Cancer Society, the most common treatments for cancer are surgery, chemotherapy, and radiation. Most people receive a combination of treatments such as surgery plus chemotherapy and/or radiation therapy due to the spread of tumor cells to near areas and/or distant areas, avoiding a successful tumor elimination by surgery (2). In most cases it is not possible to use surgery, thus an alternative is the use of cytotoxic drugs to treat cancer. Most of the chemotherapeutic drugs have similar mechanisms of action, inducing cytotoxicity in cells undergoing rapid growth and proliferation, one of the hallmarks of cancer cells (3). For example, cisplatin is mainly used to treat bladder, ovarian, and testicular cancer (4). Another example is gemcitabine, which is used to treat solid tumors such as pancreatic, breast, ovarian, and lung cancer (4).

1.2 GEMCITABINE

Gemcitabine is marketed as Gemzar® by Eli Lilly and Company. Its empirical formula is $C_9H_{11}F_2N_3O_4 \bullet HCl$ (gemcitabine HCl), with a molecular weight of 299.66 (g/mol) (5). It is soluble in water and is supplied for intravenous administration only (5). Gemcitabine was approved in 1996 by the Food and Drug Administration (FDA) as the first-line treatment for locally advanced and metastatic pancreatic cancer (6). Furthermore, it is administrated in combination with other drugs such as cisplatin, paclitaxel, or carboplatin to treat other cancers (i.e. locally advanced or metastatic non-small cell lung cancer, metastatic breast cancer, and advanced ovarian cancer; respectively) (7-9). Gemcitabine HCl (2´,2´-difluorodeoxycytidine HCl, dFdC) is a nucleoside analogue, which requires intracellular uptake, followed by sequential phosphorylation for its activation (6, 10). Because of its high hydrophilicity, it is not able to permeate plasma membranes by passive diffusion (6, 10, 11). For this reason, gemcitabine must be internalized into cells by specialized nucleoside transporters such as concentrative nucleoside transporter (hCNTs) and equilibrative nucleoside transporter (hENTs) (12, 13). However, kinetic studies demonstrated that gemcitabine is mainly incorporated by hENT1, which is critical for optimal response of gemcitabine (14). Indeed, its high expression was associated with *in vitro* chemosensitivity of gemcitabine in human pancreatic adenocarcinoma and biliary tract carcinoma cells (15). Once into the cells, gemcitabine undergoes three sequential phosphorylations that allow its activation (6, 10). The rate-limiting step of activation is mediated by enzymes such as deoxycytidine kinase (dCK), which allows the first phosphorylation of gemcitabine, followed by two more rounds of phosphorylation to produce the active metabolites of gemcitabine (i.e. 2´,2´-difluoro-2´-deoxycytidine triphosphate; dFdCTP) (6, 10). Therefore, this step is essential to the cytotoxic activity of gemcitabine (6, 10, 16). The most important mechanism of action of gemcitabine is inhibition of DNA synthesis (16). Gemcitabine is able to inhibit DNA synthesis because its triphosphate phosphorylated form (dFdCTP) is a competitive substrate of deoxycytidine

triphosphate, thus dFdCTP is incorporated into the DNA during replication, inhibiting the elongation chain (6, 10, 16). This process is known as "masked chain-termination" due to the non-terminal position of dFdCTP, avoiding the detection and repair of DNA (6, 10, 16).

In addition to its main function, gemcitabine has at least two more mechanisms of action: 1) induction of apoptosis by caspase signaling, and 2) self-potentiation through inhibition of enzymes involved in deoxynucleotide metabolism, which allow high intracellular levels of the active metabolite, improving its mechanism of action (10, 17, 18).

Gemcitabine is normally administrated as an intravenous infusion at a dose of 1000-1250 mg/m^2 given once weekly for a 3- or 4-week cycle (5). It has a short plasma circulation time (19). The elimination half-life depends on the gender and age of patients, as well as infusion duration (6). Longer infusion times are associated with higher toxicity (6). The most common toxicities associated with gemcitabine are myelosuppression, high levels of hepatoxicity, renal toxicity, thrombocytopenia, and anemia (19-21).

1.3 GEMCITABINE CHALLENGES AND LIMITATIONS

A major limitation of gemcitabine is the quick development of resistance by individual patients during treatment cycles. Gemcitabine resistance has been associated with the following factors:

1.3.1 Inhibition of nucleoside transporters

Gemcitabine is a hydrophilic drug with low membrane permeability, thus it requires a specialized transport system (i.e., nucleoside transporters) to be incorporated into the cells (11). A deficient influx of gemcitabine is associated with resistance to gemcitabine. Indeed, the absence

or low expression of hETN1, as well as hCNT3, are the most common causes of resistance in human breast and pancreatic cancer (12, 22). Thus, studies have proposed their evaluation as predictive factors for gemcitabine response (23, 24)

1.3.2 Deficiency of dCK activity.

As mentioned previously, gemcitabine is a prodrug that must undergo a series of phosphorylations to be active inside the cell, of which dCK is the rate-limiting enzyme responsible for the first phosphorylation of gemcitabine (6, 10, 25). A clinical study reported that high levels of dCK correlated with gemcitabine efficacy in pancreatic cancer, improving the overall survival in patients (26). On the other hand, regulation at the post-transcriptional level of dCK is associated with gemcitabine resistance in pancreatic cancer cells (6, 27). A study reported that overexpression of HuR in MiaPaCa2 (a pancreatic cancer cell line) correlated with a high sensitivity to gemcitabine (27). The same study also reported that the histology analysis of patients with pancreatic ductal adenocarcinoma showed a correlation between low cytoplasmic expression of HuR and poor survival due to resistance to gemcitabine compared to patients with high cytoplasmic expression of HuR, exhibiting a better prognosis (27)

1.3.3 Overexpression of ribonucleotide reductase.

Ribonucleotide reductase (RR) is a holoenzyme formed by two subunits RRM1 and RRM2 (6, 10). The main function of this enzyme is to catalyze the conversion of ribonucleotide to deoxyribonucleoside triphosphate (dNTPs), which is modulated by the levels of its catalytic subunit M2 (RRM2) (6, 10, 16, 25, 28). Studies correlated the overexpression of RRM2 with resistance to gemcitabine in pancreatic, biliary, colon, and non-small cell lung cancer (10, 15, 28-31). Indeed, *in vitro* and *in vivo* studies in pancreatic adenocarcinoma demonstrated that the suppression of RRM2 using RNA interference (siRNA) improves gemcitabine cytotoxicity,

suggesting the combination of gemcitabine and siRNA targeting RRM2 can induce strong suppression of tumor growth, increased apoptosis, and inhibition of metastasis (31).

On the other hand, resistance to gemcitabine is not only associated with these three mechanisms, and other factors might also contribute to gemcitabine resistance. For example, deregulation of genes associated with apoptosis and survival (i.e., p53, Bcl-2, Bax, and Bak) are associated with gemcitabine resistance (32, 33). In addition, studies reported that NF-κB and also HIF-1α might play a role in resistance through inhibition of gemcitabine uptake and apoptosis (10, 27, 32, 33).

Another limitation associated with gemcitabine is a short plasma half-life due to the rapid and extensive inactivation by cytidine deaminase (CDA) to its inactive metabolite 2´, 2´-difluoro-deoxyuridine (dFdU) (34). CDA is an enzyme ubiquitously expressed in the liver and blood, thus inactivation of gemcitabine mainly occurs in the liver and to a lesser extent in the blood, followed by rapid excretion in the urine (6, 34).

Considering these limitations of gemcitabine, new strategies to improve the transport and avoid its inactivation are necessary. Therefore, the focus of the next section is to discuss some examples of chemical modification at 4-(*N*)-positions to overcome the mechanism of gemcitabine resistance by protection from deamination.

1.4 CHEMICAL MODIFICATION OF GEMCITABINE AT 4-(*N*)- POSITION

Drug modifications have long been used in different anticancer drugs to overcome the limitations of the parent drug such as rapid clearance, enzymatic degradation, poor uptake, pharmacokinetic limitations, and so on. In the case of gemcitabine, chemical modification at the 4-(*N*)-position confers special protection against deamination, improving its bioavailability (6). The activation of gemcitabine occurs by the hydrolysis of the amide bond, which has a slower rate

due to its higher stability, conferring extended blood circulation (35) . Some examples of 4-(*N*)-modifications of gemcitabine are:

1.4.1 PEG-Gemcitabine.

This prodrug is formed by the conjugation of gemcitabine with poly(ethylene glycol) to improve the drug plasma half-life due to low clearance by the kidneys (36). In addition, the prevention of degradation by cytidine-deaminase increases significantly the bioavailability of PEG-gemcitabine over gemcitabine (36). Similar results were reported when Ara-C, structurally similar to gemcitabine, was conjugated at 4-(*N*)-position with PEG, improving its stability and pharmacokinetic parameters (37).

1.4.2 LY2334737

Oral administration of a prodrug of gemcitabine is achievable rather than gemcitabine (6, 38). Oral administration of gemcitabine is not feasible due to the abundant concentration of CDA in blood, liver, and gut, which metabolizes gemcitabine to dFdU (6, 34, 38). In fact, gemcitabine has a low systemic exposure due to extensive first-pass metabolism to dFdU, showing a moderate gastrointestinal toxicity when orally administrated to patients with refractory tumors (38). Therefore, amine group protection by prodrug formation can hinder the deamination site, improving the oral bioavailability (6). For instance, LY2334737 was developed by covalently linking valproic acid (VPA) via an amide bond at the 4-(*N*)-position, reporting a good chemical and enzymatic stability (39). A preclinical study demonstrated the intact absorption of LY2334737 by the intestinal epithelium, avoiding first-pass metabolism and allowing its intact delivery into the systemic circulation (40). Moreover, LY2334737 has a slow hydrolysis, leading to sustained *in vivo* release of gemcitabine (40). This study also showed that carboxylesterase 2 (CES2) was responsible for the *in vitro* hydrolysis of LY2334747 (40). A phase I study in Japanese patients

with advance solid tumors showed a good tolerability at doses up to 30 mg/day while at the 40 mg/day dose some patients reported dose-limiting toxicities (DLT) such as hepatic toxicity, thrombocytopenia, and disseminated intravascular coagulation (41). LY2334747 was also used in combination with erlotinib in a phase I study, in which the maximum tolerated dose (MTD) was 40 mg with or without daily administration of 100 mg of erlotinib in European patients with advanced solid tumors (42). The most common DLT for LY2334737 monotherapy were fatigue and high levels of transaminase, while for combination therapy the most common adverse events were fatigue, nausea, vomiting, and elevated transaminase levels (42).

1.4.3 Lipophilic prodrugs

Lipophilic derivatives of gemcitabine are another strategy used to improve blood stability and increase gemcitabine cytotoxicity (6). This strategy consists of conjugating gemcitabine with lipids such as fatty acids or phospholipids to increase its lipophilicity, as well as modify other properties of gemcitabine (35). An amide bond can be formed through the covalent conjugation of the fatty acids' carboxylic acid end position to the amine group of gemcitabine, while an ester bond may be formed by the reaction between the hydroxyl group of gemcitabine and the carboxylic acid group of phospholipids (6, 35). Some examples of fatty acids conjugated with gemcitabine will be discussed next.

As previously mentioned, a lipophilic derivative of gemcitabine can be formed by covalent conjugation of fatty acids, such as squalene acid (SQ), stearic acid (SA), or docosahexaenoic acid (DHA) (43-46). For example, squalenoyl gemcitabine (SQdFdC) is formed by the conjugation of squalene with gemcitabine and allows the formation of amphiphilic molecules that can self-organize in water as nanoassemblies (SQdFdC NA) of 100-300 nm (43). *In vitro* studies showed a higher cytotoxicity of SQdFdC NA, rather than gemcitabine, against MCF-7 (human breast carcinoma) and KB3-1 (human nasopharyngeal carcinoma) cells, and also a strong cytotoxicity against L1210k cells, a resistant phenotype of L1210wt cell with downregulation of deoxycytidine

kinase (dCK) (43, 47). Intravenous administration of SQdFdC NA exhibited strong anticancer activity against leukemia-bearing mice and solid P388 tumors, increasing survival and limiting tumor growth, respectively (48). In fact, SQdFdC NA increased the apoptosis level of cancer cell lines compared to gemcitabine (47). Moreover, a preclinical toxicity study showed a dose-dependent toxicity independent of the route of administration (i.e. intravenous or subcutaneous), reporting a similar toxicological profile to gemcitabine at equitoxic doses, while a potent anticancer activity was reported in a leukemia model with 75% long-term survivors (49). This higher efficacy of SQdFdC NA is due to the favorable modification of pharmacokinetic parameters, as well as a selective organ distribution (50). For instance, half-life and mean residence time of SQdFdC NA were greater than gemcitabine administrated as free drug (~3.9-fold and ~7.5-fold, respectively) (50). In addition, the biodistribution study showed longer body retention of SQdFdC compared to dFdC, as well as higher accumulation of SQdFdC in spleen and liver, the main organs responsible for metastasis in leukemia and other cancers (50). In addition, the anticancer efficacy of SQdFdC NA after oral administration to rats bearing large granular lymphocytic (LGL) leukemia was also investigated (43). SQdFdC NA exhibited superior anticancer activity as compared to gemcitabine, increasing the life span of rats and the number of long-term survivors (43). The factors that could explain this superior efficacy of SQdFdC NA are: 1) resistance to deamination, 2) favorable pharmacokinetics, and 3) increased accumulation in the lymphoid organs (51). Therefore, these data support the candidacy of SQdFdC NA as alternative therapy to leukemia by oral or parental route (43, 51).

4-(*N*)-stearoyl-gemcitabine (i.e. GemC18) and 4-(*N*)-lauroyl-gemcitabine (i.e. GemC12) are other examples of lipophilic gemcitabine prodrugs, which showed higher cytotoxicity activity against HT-29 colon adenocarcinoma and KB nasopharyngeal carcinoma compared to gemcitabine (44). In addition, GemC18, as well as GemC12, showed a slow metabolism in plasma, with 90 % of the intact prodrug recovered after 8 h while no more than 40% of unmodified gemcitabine was recovered after 8 h (44). GemC18 was encapsulated into liposomes to improve the *in vitro* and *in vivo* stability (44). Its encapsulation enhanced the passive diffusion across the

cell membrane, increasing the prodrug cytotoxicity in comparison with gemcitabine, which is not permeable to the cell membrane as previously mentioned (11, 13, 44, 52). Therefore, GemC18-liposome allows protection against deamination, increasing the plasma half-life of the drug, which increases the antitumor potency due to its higher tumor concentration (52).

Another example of a lipophilic derivative of gemcitabine is 4-(*N*)-docosahexaenoyl 2′, 2′-difluorodeoxycytidine (DHA-dFdC), which was synthetized by conjugation of docahexaenoic acid (DHA), an omega-3 polyunsaturated fatty acid (PUFA), to dFdC at the 4-(*N*)-position. This novel compound showed promising results such as potent cytotoxicity against several cancer cell lines (e.g. leukemia, renal, pancreatic, breast cancer cells) and strong antitumor activity against MCF-7 tumor-bearing nude mice, Panc-1 pancreatic tumors, and leukemia bearing mice as compared to gemcitabine (45, 46). In fact, DHA-dFdC was significantly more cytotoxic than dFdC in BxPC-3 cells (i.e. pancreatic cancer cells) with an IC_{50} of 100,000-fold smaller than dFdC (46). These data show the potency of DHA-dFdC at significant lower dose than dFdC that might help to reduce the toxic effect of dFd on normal cells (45). Therefore, *in vivo* studies showed that DHA-dFdC significantly inhibited Panc-1-luc tumor growth while equimolar dose of dFdC did not significantly inhibit the tumor growth (46). These data demonstrated the strong antitumor activity of DHA-dFdC over dFdC due to Panc-1 tumor cells are known to be resistance to dFdC, thus DHA-dFdC might a new potent drug derivate from dFdC that migh a potential candidate to treat pancreatic cancer resistant to dFdC (46). Moreover, mice genetically engineered to spontaneously develop pancreatic ductal adenocarcinoma treated with DHA-dFdC showed greater survival than untreated mice (46). The potent antitumor activity of DHA-dFdC might be related to its DHA portion, which can increase drug transportation across tumor cell membranes (45). Furthermore, studies relate the use of natural fatty acids by tumor cells as a source of energy and biochemical precursor supply in several processes (53-55). In fact, various drugs (e.g. paclitaxel, doxorubicin, 10-hydroxycamptothecin) have been conjugated with DHA to improve their tumor targeting, leading to greater drug concentration in the tumor and, as a consequence, reducing toxicity in normal tissues (35, 54, 56). For instance, DHA-paclitaxel (or Taxoprexin) showed preferential

uptake by tumor compared to normal tissue at preclinical levels (35, 56). DHA conjugated to paclitaxel changes the pharmacokinetic profile of paclitaxel, increasing the concentration and retention of DHA-paclitaxel that slowly might cleave and convert into paclitaxel (56). Similar results were found in phase I clinical trials where DHA-paclitaxel showed higher half-life and slow clearance compared to paclitaxel (57). Therefore, the tumor targeting strategy might explain the greater potency of DHA-dFdC in several tumor models, but more studies related with the formulation of DHA-dFdC are necessary due to its chemical instability in solution and poor water solubility, as both properties might affect its performance in clinical studies. Due to the promising results of DHA-dFdC, the improvement of its formulation is one of the main purposes of this dissertation and will be discussed later.

In terms of toxicity, DHA-dFdC was well-tolerated at preclinical levels but this study will be discussed in detail in the next chapter of this dissertation.

1.4.4 Advantages of lipophilic prodrug of gemcitabine

As discussed in the previous section, the lipophilic prodrugs of gemcitabine offer several advantages over gemcitabine. For instance:

1.4.4.1 Lipophilic prodrugs can improve the oral bioavailability of gemcitabine.

Lipid-gemcitabine conjugation at 4-(*N*)-position protects from extensive deamination in the liver and gut, as previously mentioned. An example of an oral gemcitabine prodrug is SQdFdC NA , which showed strong antitumor activity against a leukemia rat model at preclinical levels (51). In general, lipid-conjugation increased the lipophilicity of the parental drug, which can improve the membrane cell interaction (6, 35, 44). In addition, lipid-drugs can use the lymphatic system, avoiding the first pass-metabolism, and contribute enhanced oral bioavailability (35, 43, 58). However, the lymphatic transport will depend on the lipophilicity of the drug. Thus, a lipid-

drug with log*P* higher than 5 will be transferred directly into the lymphatic system while a lipid-drug with log*P* lower than 5 will be transported to the blood circulation (35).

1.4.4.2 Improve tumor targeting and as a consequence reduce toxicity.

Most of the examples previously discussed showed higher tumor targeting. For example, biodistribution of DHA-dFdC showed higher concentrations in mouse pancreas compared to other organs (i.e. liver, kidney, spleen, lung, and heart), one hour after intravenous administration (46). Furthermore, DHA-dFdC inhibited tumor growth in nude mice with orthotopic pancreatic tumors, as well as improved survival in transgenic mice that spontaneously developed pancreatic tumors (46). This evidence suggests tumor targeting of DHA-dFdC against pancreatic cancer.

Lipophilic prodrugs of gemcitabine reduce drug toxicity due to their strong tumor targeting. In addition, the prodrug is inactive until the hydrolysis of the amide bond occurs in the tumor cells, minimizing the exposure of normal organs to the active drug. Cathepsins are lysosomal enzymes overexpressed in cancer cells, which are mainly responsible for prodrug activation by cleavage of amidic-linkage (59). Cathepsin B (a cysteine protease) and cathepsin D (an aspartic protease) are the main enzymes responsible for SQdFdC NA and GemC18 hydrolysis, converting the prodrug into the active gemcitabine (43, 44).

1.4.4.3 Lipophilic prodrugs of gemcitabine can be encapsulated into nanoparticles.

Lipophilic drugs have greater drug loading into carriers that contain lipophilic components, exhibiting minimal drug leakage (35). The most common carriers for lipophilic drugs are liposomes, polymeric micelles, polymeric nanoparticles, and solid lipid nanoparticles. SQdFdC NA, as well as GemC18, have been incorporated into delivery carriers, improving their antitumor activity and their pharmacokinetic parameters (6, 35). Indeed, the encapsulation of GemC18 into solid lipid nanoparticles allows its oral administration, overcoming detrimental effects of the

formulation (60). Due to its low solubility, GemC18 was dissolved in oil for oral administration in mice, which resulted in significant weight loss and signs of distress (60). The adverse effects were attributed to the GemC18-oil formulation, since gavaging with oil alone was well-tolerated by mice (60).

As mentioned before, the poor water solubility of lipophilic drugs (e.g. GemC18 and dFdC-DHA) is the main problem for drug formulation, restricting their administration. Furthermore, the formulation (i.e. lipophilic prodrug in the vehicle) might induce side effects, like GemC18 in oil. Additionally, the lipophilic prodrug might exhibit chemical instability, like dFdC-DHA. Thus, the encapsulation of these lipophilic prodrugs into nanoparticles has emerged as an alternative technology to overcome these problems. Furthermore, nanoparticles may improve their antitumor activity as well as their pharmacokinetic parameters.

1.5 NANOTECHNOLOGY AS A TOOL TO IMPROVE GEMCITABINE LIPOPHILIC DERIVATIVES PROPERTIES

Nanotechnology may improve the therapeutic effectiveness and safety profile of chemotherapeutics, such as lipophilic derivatives of gemcitabine. Nanoparticles provide extra protection from deamination, thus prolonging the drug blood circulation (6, 35, 61-63). In addition, the incorporation of hydrophilic polymers (e.g. polaxamers or PEG) into the shell increases the drug half-life, reducing aggregation and immunogenicity (6, 63, 64). Cancer cells are able to develop drug resistance that restricts the therapeutic efficacy of chemotherapeutic drugs. The use of nanoparticles as a carrier delivery system might overcome drug resistance and also increase the drug cytotoxicity (3, 63). In addition, coating nanoparticles with specific ligands (e.g. folic acid, transferrin, EGF, lectins, VEGF, VCAM-1, and so on) that are overexpressed on cancer cells can improve their cell uptake, reducing the drug toxicity and increasing its antitumor activity (6, 35,

63, 65). In this section, some examples of lipophilic derivates of gemcitabine loaded into nanocarriers and their main advantages are discussed.

1.5.1 Liposomes

Liposomes are small spherical vesicles that are mainly composed by biocompatible and biodegradable phospholipids that can form one or more bilayers, mimicking the cell membrane structure (66). Liposome structure is organized as an enclosed compartment on which phospholipid bilayers divide an aqueous medium from another. This structure allows the entrapment of hydrophilic drugs in the aqueous compartment while lipophilic drugs are incorporated in the lipid membrane (35). Some examples of liposomes approved by the FDA are Doxil® and Marqibo®, in 1995 and 2012, respectively (67). Doxil® is a doxorubicin hydrochloride (HCl) liposome injection used to treat AIDS-related Kaposi's sarcoma, breast cancer, ovarian cancer, and other solid tumors (67). Marqibo® is a vincristine sulfate liposome injection used to treat adult Philadelphia chromosome (Ph)-negative acute lymphoblastic leukemia (ALL) (67).

Liposomes present various advantages such as: 1) improved efficacy and therapeutic index of drugs, 2) protection of the loaded drug from inactivation following intravenous administration, 3) use of biocompatible and biodegradable materials, 4) improved safety due to non-toxicity, 5) flexibility to combine with site-specific ligands to achieve active targeting, 6) increased concentration of the encapsulated drug in the tumor, and 7) low risk of nonspecific toxicity (61, 66, 67). However, liposomes also present various disadvantages such as 1) low solubility, 2) short half-life, 3) leakage and fusion of encapsulated drugs, and 4) low stability, limiting their use in cancer therapy (61, 67). A good example of these limitations is gemcitabine-loaded liposomes (66). Due to its hydrophilic nature, gemcitabine is completely loaded into the aqueous compartment of liposomes and rapidly diffuses out of liposomes (68). To overcome this disadvantage, the use of lipophilic prodrugs of gemcitabine have emerged as an alternative to encapsulate into liposomes (44, 66). For example, 4-(*N*)-acyl derivative prodrug of gemcitabine

loaded in liposomes showed a better stability and higher encapsulation efficiency (EE) than gemcitabine-loaded liposomes (44). The EE is mainly affected by the length of saturated 4-(*N*)-acyl chain, the nature of phospholipids, and the presence of cholesterol (44). Indeed, 4-(*N*)-stearoyl-gemcitabine (GemC18) and 4-(*N*)-lauroyl-gemcitabine (GemC12) in liposomes composed by DSPC/DSPG 9:1 showed greater EE, at ~98% and ~94%, respectively (44). Furthermore, GemC18 liposomes exhibited better *in vitro* stability on storage than GemC12 liposomes (44). Moreover, the encapsulation of GemC18 into liposomes provides an additional protection against deamination, improving its pharmacokinetic behavior (6, 44). For example, GemC18 liposomes exhibited higher half-life and AUC than gemcitabine when intravenously administered (3.5-fold and 50-fold, respectively), resulting in strong antitumor activity due to its higher tumor accumulation (44, 52).

1.5.2 Polymeric micelles

Polymeric micelles are small sized (< 200 nm) drug delivery systems composed of amphiphilic polymers that self-assemble into core-shell structured nanocarriers, which include a hydrophilic shell and a hydrophobic core (35, 69). This core-shell structure allows the encapsulation of hydrophobic drugs into the core by no covalent interactions (35). In addition, the hydrophilic shell can increase their circulation in the blood and sterically stabilize, preventing aggregation and protein adhesion (69, 70). Polymeric micelles might contain a broad spectrum of polymers, but the use of biodegradable and biocompatible polymers is required to ensure their safety as nanocarriers (70-72).

One of the most critical requirement of micelles is good stability of drug loading to achieve an optimal tumor targeting (69). Lipophilic prodrug strategy is a promising alternative to improve drug loading into micelles, minimizing their drug release before reaching the tumor site (35, 69). For example, doxorubicin-palmitic acid (DOX-PA) was synthesized by conjugation of doxorubicin with palmitic acid through an acid pH-responsive hydrazone linker, which is stable at

neutral pH (69). DOX-PA loaded into DSPE-PEG showed a higher encapsulation efficiency (i.e. ~ 100 %) and good stability (69). In addition, this formulation did not show drug expulsion after 3 weeks of storage at 4°C (69). Therefore, lipid conjugation with DOX might improve its lipophilicity, improving its encapsulation efficiency (35). Moreover, the lipid portion of DOX-PA can enhance compatibility and interaction of DOX with the lipid core of DSPE-PEG micelle, inducing a higher stability of the drug loaded into the micelles (69).

GemC18 is another example of a lipophilic prodrug loaded into micelles. GemC18 loaded into poly(ethylene glycol)-poly(D,L-lactide) (PEG-PLA) polymeric micelles exhibited a higher encapsulation, sustained release, and higher cytotoxicity compared to Gem and GemC18 solution against pancreatic cancer cell lines (i.e. Panc-1, and AsPC-1 cells) (72). *In vivo* and *in vitro* GemC18 loaded into DSPE-PEG/tocopherol-PEG 100-succinate (TPGS) mixed micelles increased drug stability by protection against deamination, improving its pharmacokinetic profile (73). Indeed, after intravenous administration, GemC18-loaded DSPE-PEG/TPGS micelles showed 2-5-fold higher plasma concentration, 3-fold greater systemic exposure (AUC), and lower volume of distribution at the steady state (Vd,ss), suggesting slow release of GemC18 from the mixed micelles and increased circulation in the bloodstream (73). Furthermore, this formulation exhibited stronger antitumor activity than free drug against BxPC-3 tumor bearing mice (73).

Acid-sensitive micelles is another approach to improve tumor targeting due to the acidic extracellular environment of the tumor tissue (pH ~6.8), compared with surrounding normal tissues (74). GemC18 loaded into pH-sensitive micelles were prepared by conjugation of PEG with a hydrophobic stearic acid derivate (C18) using an acid-sensitive hydrazone bond (PHC) (75). GemC18 carried by pH-sensitive micelles exhibited stronger *in vivo* antitumor activity compared to acid-insensitive micelles, gemcitabine HCL in solution, or GemC18 in solution against B16-F10 melanoma-bearing mice (75). The lysosomal delivery of GemC18 by micelles confers various advantages such as longer blood circulation time, higher tumor accumulation, and protection from deamination, and as consequence increased antitumor activity of GemC18 (75). In fact, this formulation was able to overcome the tumor cells resistant to gemcitabine through inhibition of

the expression of RRM1 and increased the level of gemcitabine triphosphate (dFdCTP), which can explain the greater antitumor activity of this formulation (76).

1.5.3 Polymeric nanoparticles

Polymeric nanoparticles (PNPs) are biodegradable colloidal systems, exhibiting various alternatives as drugs carriers, such as: 1) absorbed in the polymeric matrix, 2) entrapped into the core surrounded by the polymeric matrix, or 3) conjugated either within or onto their surface (71, 77). The main attributes for selection of the correct polymer are easy manufacturing, biodegradability, and extended drug release (71, 77). As with polymeric micelles, the shell function provides drug protection against enzymatic degradation. For instance, polyvinyl alcohol (PVA), polyethylene glycol (PEG), polylactic acid (PLA), poly(D,L- lactide-co-glycolic) acid (PLGA), monomethoxy poly-(ethylene glycol) (mPEG), polysorbate and Vitamin E D-alpha tocopheryl polyethylene glycol 1000 succinate (TPGS) are common polymers used in PNPs (71).

PNPs also have been used as carriers for lipophilic prodrugs such as UDCA-AZT (a prodrug of zidovudine), GemC18, and SN-38 (an inhibitor of topoisomerase I) (78-81). PLGA and/or PEG are common polymers used to deliver lipophilic prodrugs in PNPs (6, 71, 79, 81). For example, PEG-PLGA NPs were used to deliver SN-38, which is very unstable and incompatible with various carriers, and exhibited greater stability, longer half-life, and promoted its tumor accumulation (78). This formulation takes advantage of the leaking tumor structure, increasing the drug accumulation through the enhanced permeability and retention effect (EPR). On the other hand, active targeting also has been used to improve the antitumor effect of PNPs. For example, several studies demonstrated the over-expression of the epidermal growth factor receptor (EGFR) in various human tumor cell lines such as head and neck cancer cells, breast cancer cells, pancreatic cancer cells, and so on (82, 83). Thus, its ligand (EGF) was used to target GemC18-NPs (EGF-GemC18-NPs), showing stronger anti-tumor activity than GemC18-NPs (65).

1.5.4 Solid lipid nanoparticles

Solid lipid nanoparticles (SLNs) are colloidal systems with a submicron range of diameter (50-1000 nm), which offer an alternative to traditional colloidal carriers, such as emulsions, liposomes, and polymeric micro- and nanoparticles (84, 85). SLNs structure contain a solid lipid core surrounded by a monolayer phospholipid shell (86). The lipophilic portion of the phospholipid is embedded in the lipid matrix (86). SLNs are prepared using lipids solid at room and body temperature (i.e. mono-, di-, and triglycerides, fatty acids, waxes and steroids) stabilized by surfactant(s) (i.e. phospholipids, Poloxamers, and Polysorbates) (62, 86). SLNs can encapsulate both lipophilic and hydrophilic drugs, thus the drug encapsulation will depend on three factors: 1) type of solid lipid selected, 2) method of preparation, and 3) polymorphic change in lipid crystal (86, 87). This formulation exhibits several advantages such as improved drug stability, easy sterilization, enhanced bioavailability, chemical protection of labile drugs, easier and cheaper manufacturing than PNPs, low toxicity, and controlled release (62, 84, 86, 88). Furthermore, most of the lipids and surfactants used to prepare SLNs are approved by the FDA and have GRAS (Generally Recognized As Safe) status (62). In addition, SLNs manufacturing allows their administration through different routes such as parental (i.e. intravenously, intramuscularly or subcutaneously), oral, rectal, ophthalmic, and topical (62, 89-95).

SLNs have been proposed as carriers for lipophilic prodrugs due to their efficient encapsulation into the lipid core, improved drug stability by protection from enzymatic degradation, as well as delayed drug release (62, 88). In addition, tissue targeting is an additional benefit conferred by SLNs (3). For instance, 3', 5'-dioctanoyl-5-fluoro-2'-deoxyuridine (DO-FUdR), a lipophilic prodrug derived from 5-fluoro-2'-deoxyuridine (FUdR), was incorporated into SLNs, showing *in vitro* extended drug release and *in vivo* specific brain targeting after intravenous administration (96). In fact, AUC and half-life values of DO-FUdR-SLN were higher in the brain compared to DO-FDUdR (~2.06-fold and ~1.49-fold, respectively) (96). Moreover, SLNs can increase drug cytotoxicity due to their faster internalization into cells, followed by the drug's release from SLNs inside of the cells (97). For example, SN38 was incorporated into SLNs based

on Compritol 888 ATO, exhibiting higher cytotoxicity against breast cancer cell lines (i.e. MCF7) compared to SN38 in solution (98). In addition, other SLN formulations of SN38 based on Compritol 888 ATO and Precirol showed greater *in vivo* and *in vitro* antitumor activity against C-26 tumor bearing mice (i.e. colorectal carcinoma tumor) compared to the commercially available parent drug (i.e. irinotecan) (99). Indeed, SLN-SN38 improved mice survival at lower doses (i.e. 20 mg/kg) than irinotecan MTD (i.e. 50 mg/kg), decreasing the toxicity associated with higher concentrations (99).

SLNs also can be orally administered as previously described (90, 91). Oral administration is considered one of the most traditional routes of drug delivery, offering various advantages such as convenient, comfortable, painlessness, easy self-administration, high patient compliance, and low cost (86). However, this route has several limitations related to physicochemical properties of the drug such as poor solubility and chemical instability in the gastrointestinal tract (GI), low permeability, and rapid metabolism, which decrease the oral bioavailability (58, 86). SLNs have emerged as alternative drug delivery systems for oral administration due to their ability to improve the oral bioavailability through their controlled release, protection against enzymatic degradation in the GI tract, as well as site-specific drug delivery (86). In fact, SLNs have been used successfully as oral delivery systems to improve the oral bioavailability of lipophilic drugs such as docetaxel, paclitaxel, cyclosporine A, vinpocetine, lopinavir, quercetin, and so on (86, 100-104). Furthermore, the nature of the shell coating on SLNs might allow their direct absorption by the lymphatic system, avoiding the first past metabolism (86). For example, wheat germ agglutinin (WGA)-coated SLNs improved the oral bioavailability of paclitaxel due to WGA linked N-acetyl-D-glucosamine and sialic acid localized on intestinal surface cells (101, 105). Moreover, SLNs coated with D-α-tocopheryl poly(ethylene glycol) succinate (TPGS) enhanced the intestinal absorption and bioavailability of docetaxel, due to its potent Pgp-inhibiting activity (100). Another example of coating modification are PEGylated SLNs, in which PEG acts as a potent mucus-penetrating agent, overcoming the mucus barrier and improving drug transport across the gastrointestinal epithelia (106).

GemC18-SLNs is an example of lipophilic prodrug loaded into SLNs for oral administration. Previously, GemC18-SLNs, prepared with soy lecithin, glycerol monostearate (GMS), Tween 20, and DSPE-PEG (2000), showed stronger antitumor activity against mouse models of lung and pancreatic cancer than equimolar doses of free gemcitabine given by intravenous administration (107). Similarly, oral administration of GemC18-SLNs exhibited a higher efficacy against pre-established lung tumors (i.e. mouse TC-1 or LCC lung cancer cells) compared to free gemcitabine HCL or GemC18 alone at an equimolar dose (60). Moreover, GemC18-SLNs showed a higher absolute oral bioavailability than that of gemcitabine (~70 % based on $AUC_{0\text{-}24h}$), which might help to explain the GemC18-SLNs ability to inhibit tumor growth, as well as increase mice survival in pre-established lung tumor models (60). Immunohistostaining of LCC tumor suggested that the mechanism of action of oral GemC18-SLNs was inhibition of tumor proliferation and angiogenesis, while increasing cell apoptosis (60).

Nanoparticles can reach the tumor through passive targeting, in which the defective and leaky vasculature tumor architecture allows their passive diffusion into cancer cells (63). Furthermore, the inefficient lymphatic drainage of the tumor leads to nanoparticles' extravasation and retention in the interstitium of tumors, a phenomenon known as ″enhanced permeability and retention″ (EPR) (63, 108). However, passive targeting is determined by the tumor microvasculature (e.g. degree of tumor vasculature and angiogenesis), and nanoparticle properties (e.g. size, shape, and surface charge) (63). The ideal nanocarrier has at least three characteristics: 1) a size between 10-100 nm to avoid filtration by the kidney (> 10 nm) and capture by the liver (< 100 nm) (63); 2) a neutral or anionic charge to avoid renal elimination; and 3) a long circulation time to evade the reticule-endothelial system (63). Thus, SLNs that have these properties might take advantage of the EPR to be incorporated into the tumor cells.

Considering this evidence, SLNs were selected as a carrier for a DHA-dFdC, a lipophilic gemcitabine derivate, to improve its solubility, enhance its chemical stability, modify its pharmacokinetic parameters, as well as improve its antitumor activity.

1.6 CONCLUSION

Lipophilic gemcitabine is a promising strategy to overcome the mechanisms of resistance to gemcitabine. This strategy allows for the modification of the pharmacokinetic profile and reduction in the systemic toxicity. Lipophilic derivatives of gemcitabine, such as GemC18, SQdFdC NA, and DHA-dFdC, showed a favorable pharmacokinetic profile, low toxicity, and strong antitumor activity compared to gemcitabine. Moreover, tumor targeting is a property exhibited by lipid-drug conjugates such as lipophilic derivatives of gemcitabine. The pancreatic tumor targeting exhibited by DHA-dFdC explained its potent efficacy against pancreatic cancer compared to gemcitabine. Thus, the tumor targeting of DHA-dFdC can be exploited to treat pancreatic cancer. However, the strong antitumor activity exhibited by lipophilic derivatives of gemcitabine, like DHA-dFdC, was obtained at the expense of their solubility. The encapsulation of lipophilic derivatives of gemcitabine into nanoparticles can improve their solubility. Due to its lipophilic nature, SLNs might the best candidate as a drug delivery system to encapsulate lipophilic derivatives of gemcitabine, offering better encapsulation into the lipid core and sustained released, increasing their therapeutic effects with minimal toxicity, and protecting from chemical and enzymatic degradation. Furthermore, SLNs offer the possibility to exploit the oral route of administration, preferred by patients, physicians, and the health insurance industry due to convenience and lower costs. Thus, SLNs are a potential carrier for lipophilic drugs with low solubility and chemical instability such as DHA-dFdC.

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Chapter 2: Preclinical Evaluation of the Short-Term Toxicity of 4-(*N*)-Docosahexaenoyl 2´, 2´- Difluorodeoxycytidine (DHA-dFdC)[1]

2.1 ABSTRACT

Purpose: This study was designed to test the short-term toxicity of DHA-dFdC in a mouse model and its efficacy in a mouse model of leukemia at or below its repeat-dose maximum tolerated dose (RD-MTD).

Method: A repeat-dose dose-ranging toxicity study was designed to determine the tolerability of DHA-dFdC when administered to DBA/2 mice by intravenous (i.v.) injection on a repeat-dose schedule (i.e. injections on days 0, 3, 7, 10, and 13). In order to determine the effect of a lethal dose of DHA-dFdC, mice were injected i.v. with three doses of DHA-dFdC at 100 mg/kg on days 0, 3, and 5 (i.e. a lethal-RD). The body weight of mice was recorded two or three times a week. At the end of the study, major organs (i.e. heart, liver, spleen, kidney, lung, and pancreas) of mice that received the lethal-RD or RD-MTD were weighed, and blood samples were collected for analyses. Finally, DHA-dFdC was i.v. injected into DBA/2 mice with syngeneic L1210 mouse leukemia cells to evaluate its efficacy at or below RD-MTD.

Results: The RD-MTD of DHA-dFdC is 50 mg/kg. At 100 mg/kg, a lethal-RD, DHA-dFdC decreases the weights of mouse spleen and liver and significantly affected certain blood parameters (i.e. white blood cells, lymphocytes, eosinophils, and neutrophil segmented). At or below its RD-MTD, DHA-dFdC significantly prolonged the survival of L1210 leukemia-bearing mice.

Conclusion: DHA-dFdC has dose-limiting toxicity, affecting mainly spleen at a lethal-RD. At or below its RD-MTD, DHA-dFdC is effective against leukemia in a mouse model.

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2.2 INTRODUCTION

Gemcitabine (2`, 2`-difluorodeoxycytidine, dFdC) is a nucleoside analogue approved for the treatment of solid tumors such as pancreatic, non-small lung, breast, and ovarian cancers (1-5). However, many factors limit its use in cancer treatment, such as its short half-life and tumor cell development of resistance (6, 7). Data from clinical studies showed a mild to moderate toxicity of gemcitabine (1, 8). The most common side effects are myelosuppression, high levels of hepatoxicity, renal toxicity, thrombocytopaenia, and anemia (1, 8, 9).

Docosahexaenoic acid (DHA) is a natural omega-3 polyunsaturated fatty acid (PUFAs) with six cis double bonds (10). Since DHA cannot be synthesized by mammals, it is obtained from dietary sources such as fatty cold-water fish and fish oils (10). Data from many studies suggested that DHA has anticancer activity. For example, DHA has an inhibitory effect on breast cancer cells lines (11), induces apoptosis in breast and colon cancer cell lines (12), and inhibits angiogenesis through the inhibition of important angiogenic mediators (e.g. vascular endothelial growth factor, cyclo-oxygenase 2, nitric oxide, matrix metalloproteinases, etc.) (13). In addition, there are reports that DHA is able to inhibit tumor cell invasion and metastasis (14, 15), and data from a recent study suggested that the antitumor activity of DHA may be related to its ability to increase drug transportation across tumor cell membrane (16). Toxicity study in animals and human demonstrated that the consumption of DHA is safe. For example, data from a 90-day subchronic toxicity study showed that DHA is safe in rats at 0.5 and 1.25 g/kg body weight/day (17) . Clinical studies of DHA supplementation in adults did not show adverse effects in lipid levels (18, 19), platelet function (20, 21), or immune function (22, 23).

In spite of the promising results of the antitumor activity of omega-3 PUFAs in cell culture and in animal models, data from clinical studies do not support an anti-neoplastic activity by omega-3 PUFAs. Most clinical trials supported the role of PUFAs in cancer prevention, as adjuvants in improving chemotherapy, and preventing cachexia (24-30). For example, VITAL

cohort study reported that the incidence of pancreatic cancer was inversely related to the uptake of DHA (24). Another study showed that the supplementation of omega-3 PUFAs such as eicosapentaenoic acid and DHA through parental nutrition improves hepatic and pancreatic function in patients with major abdominal surgery for pancreatic or gastrointestinal cancer (31). This improvement has been associated with the role of omega-3 PUFAs in inflammation. For example, the use of gemcitabine plus i.v. omega-3 fatty acid rich lipid emulsion (e.g. Lipidem®) in pancreatic cancer patients improves their outcomes through reduction in circulating pro-angiogenic platelet-derived growth factor and fibroblast growth factor and pro-inflammatory factors (e.g. interleukin-6 and -8) (26, 27).

Previously, in an effort to exploit the antitumor activity of both gemcitabine and DHA, we synthesized a new compound, DHA-dFdC, by covalently conjugating DHA to dFdC at the 4-*N* position of dFdC (32). DHA-dFdC is a pale yellow waxy solid with a melting point of 96º C. It is poorly soluble in water (i.e. 25.2 ± 11.2 μg/ml) and has a log*P* value of 2.2 ± 0.3 (32). DHA-dFdC has potent cytotoxicity against a broad-spectrum of human and mouse tumor cell lines and showed significant antitumor activity in several mouse models of pancreatic cancer, including Kras-Ink4a genetically-modified mice and nude mice with orthotopically implanted human Panc-1 tumor cells (32). Importantly, in both tumor cells in culture and in animals, DHA-dFdC is significantly more effective than the molar equivalent DHA and dFdC physically mixed (32), suggesting a unique mechanism of antitumor activity by the DHA-dFdC conjugate. In the present study, we tested the short-term toxicity of DHA-dFdC in healthy DBA/2 mice by identifying its RD-MTD, a lethal-RD, and the major organ(s) and blood and serum parameters affected by DHA-dFdC at the lethal-RD. Finally, the antitumor activity of DHA-dFdC at or below its RD-MTD was also evaluated in DBA/2 mice with syngeneic leukemia to confirm that DHA-dFdC is active at or below its RD-MTD.

2.3 MATERIALS AND METHODS

2.3.1 Synthesis and formulation of DHA-dFdC

DHA-dFdC was synthesized following our previously reported conjugation scheme (32). The purity of the resultant DHA-dFdC was confirmed by NMR and Mass Spectrum analyses. DHA-dFdC was dissolved in a vehicle solution that consists of Tween 80 (10%, w/v), ethanol (5.2 %, v/v), and mannitol (5%, w/v) in water.

2.3.2 Cell lines

L1210 cells (mouse leukemia cell line) were from the American Type Culture Collection (Manassas, VA). Cells were grown in DMEM supplemented with 10% horse serum, 100 U/ml of penicillin, and 100 mg/ml of streptomycin. All cell culture medium and reagents were from Invitrogen (Carlsbad, CA).

2.3.3 Short-term repeated dose toxicity studies

The animal protocol was approved by the Institutional Animal Care and Use Committee at The University of Texas at Austin. Toxicity studies were performed using healthy DBA/2 mice (5-6 weeks) from Charles River Laboratories (Wilmington, MA). For all the toxicity studies, a mouse weight loss of ≥ 20% of its initial body weight on day 0 was considered as the end point (33, 34).

Initially, a single dose acute toxicity study was completed. Mice (n = 6, half male, half female) were intravenously (i.v.) injected once with DHA-dFdC at 85 mg/kg or 100 mg/kg. Untreated mice were used as a negative control. Mice were regularly monitored for overall health, and body weight was measured on days 3 and 6. On days 6, mice in the 100 mg/kg dose group and the untreated group were euthanized; major organs (i.e. heart, kidneys, liver, spleen, pancreas, and

lung) were collected and weighed. Experimental data for organ weights were expressed as the relative organ weight calculated as: (organ weight (g)/body weight (g)) x 100%. The percent of body weight change was similar in both treated groups, and there is not any significant difference in the relative organ weights in mice that received 100 mg/kg of DHA-dFdC or were left untreated (data not shown). An i.v. bolus dose of more than 100 mg/kg of DHA-dFdC was not attempted due to the limited solubility of DHA-dFdC in the vehicle solution, prompting us to focus on testing the toxicity of the DHA-dFdC on a repeat-dose schedule only.

A five-dose schedule (e.g. injection on days 0, 3, 7, 10, and 13) was adopted for short-term repeated dose toxicity studies. Mice (n = 8, half male, half female) were i.v. injected with DHA-dFdC at different doses: 70 mg/kg, 60 mg/kg, 53 mg/kg, 50 mg/kg, 40 mg/kg, or 0 mg/kg (i.e. vehicle control). An additional group of mice was left untreated as a negative control. Mice were regularly monitored for overall health, body weight change, and survival until 2 weeks after the last injection. The body weight of mice was recorded on days 0, 3, 7, 10, 13, 17, 21, 24, 28, and 31.

To identify a lethal-RD of DHA-dFdC, mice (n = 8, half male, half female) were i.v. injected with 100 mg/kg of DHA-dFdC three times on days 0, 3, and 5. Again, this dose was selected due to the limited solubility of DHA-dFdC in the vehicle solution. Mice in the control groups were left untreated or i.v. injected with vehicle solution (i.e. 0 mg/kg of DHA-dFdC). Mice were monitored every day, weighed on days 0, 2, 4, and 6, and euthanized on day 6.

2.3.4 Effect of DHA-dFdC at RD-MTD or a lethal-RD on major mouse organ weights and blood and serum parameters

Two experiments were carried out. In the first experiment, DBA/2 mice (n = 8, half male, half female) were i.v. injected with DHA-dFdC at 50 mg/kg on days 0, 3, 7, 10, and 13. As a control, another group of mice were left untreated. Five days after the last injection, mice were euthanized. Major organs were harvested, weighed, and reported as the weights relative to the

body weight of individual mouse. Blood samples were collected and shipped to IDEXX Laboratories (Sacramento, CA) for blood and serum parameter analyses.

In another experiment, mice (n = 8, half male, half female) were i.v. injected with DHA-dFdC at 100 mg/kg on days 0, 3, and 5. As controls, mice were either left untreated or i.v. injected with the vehicle solution alone. On day 6 mice were euthanized. Major organs were harvested and weighed. Blood samples were collected and shipped to IDEXX Laboratories for blood and serum parameter analyses.

2.3.5 Evaluation of the antitumor activity of DHA-dFdC in a mouse model of leukemia

Female DBA/2 mice (5-6 weeks) were intraperitoneally (i.p.) injected with the syngeneic L1210 leukemia cells (1 x 10^5) on day 0 to establish a mouse leukemia model (35, 36). Mice were then randomized into groups (n = 7) of untreated, vehicle alone, 1 x RD-MTD (i.e. 50 mg/kg), ¾ x RD-MTD (i.e. 37.5 mg/kg), and ½ x RD-MTD (i.e. 25 mg/kg), i.v. injected on days 1, 4, 8, 11, and 14, and monitored daily for overall health, weight change and survival (37). A mouse weight gain ≥ 60% of its initial body weight on day 0 was considered the end point (33, 34). Experimental data were reported as the mean survival time and T/U, which is defined as the ratio of the mean survival time of the treated group (T) divided by the mean survival time of the untreated group (U).

2.3.6 Statistical analysis

All statistical analyses involving comparing two groups were completed using Student´s t-test. For comparison of more than two groups, one-way ANOVA followed by a Bonferroni post hoc test was used to determine statistical significance between groups. Mouse survival data were analyzed using the Log-rank (Mantel-Cox) test to determine the level of significance. All of the

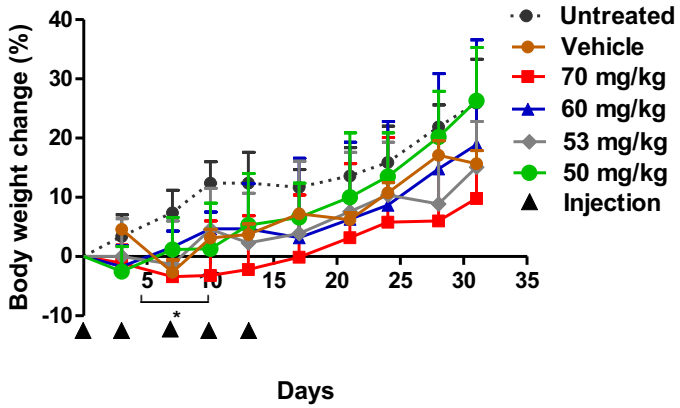
analyses were performed with GraphPad Prism (GraphPad Software, Inc., La Jolla, CA). Data shown are mean ± S.D., and a p value of ≤ 0.5 is considered significant.

2.4 RESULTS

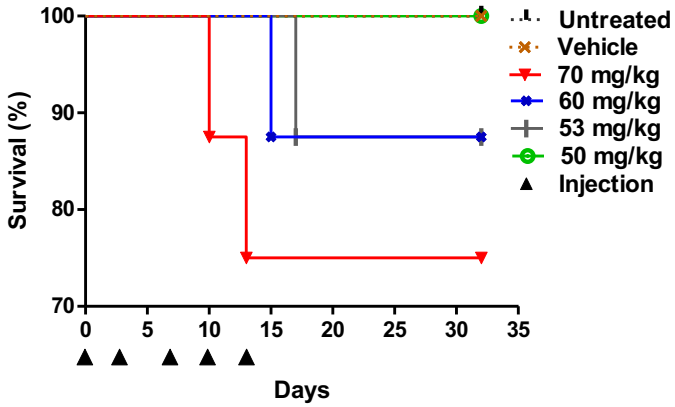
2.4.1 Identification of the RD-MTD of DHA-dFdC in healthy mice

A repeat-dose range finding study was performed to identify the RD-MTD of DHA-dFdC. Mice were i.v. injected with DHA-dFdC at various doses on days 0, 3, 7, 10, and 13. As shown in Fig. 1A, the highest dose tested (i.e. 70 mg/kg) caused significant body weight loss in mice, as compared with untreated groups (e.g. $p < 0.05$ vs. Untreated, from day 5 to day 10). In addition, in the 70 mg/kg, 60 mg/kg, and 53 mg/kg groups, some mice reached the end point (i.e. body weight loss of ≥ 20%) (Fig. 2.1B). On the other hand, DHA-dFdC at 50 mg/kg did not cause any mortality or body weight loss of ≥ 20% (Fig. 2.1A and 2.1B), and the mean mouse body weight changes did not show any difference when compared with the untreated group (Fig. 2.1A). Moreover, at 40 mg/kg, DHA-dFdC did not cause mortality, body weight loss of ≥ 20%, or any sign of toxicity in a separate experiment (Fig. 2.1C). Therefore, 50 mg/kg is considered the RD-MTD of DHA-dFdC in DBA/2 mice when administered intravenously.

(A)



(B)



(C)

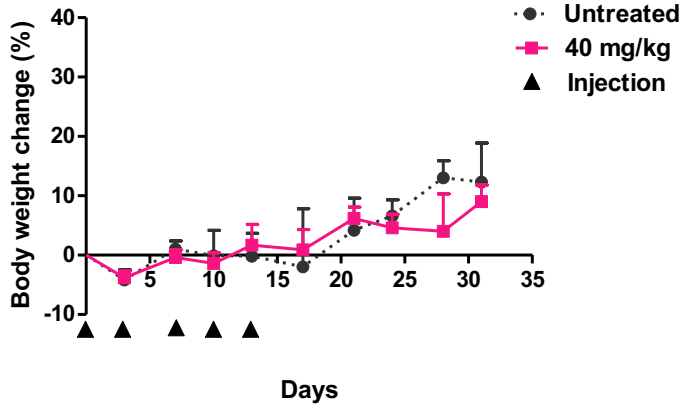


Fig. 2.1 Identification of the RD-MTD of DHA-dFdC following five i.v. injections (on days 0, 3, 7, 10, and 13 (▲)) in healthy DBA/2 mice (n = 8). (A) Percent of body weight change (* $p < 0.05$, 70 mg/kg vs. Untreated, one-way ANOVA followed by a Bonferroni post hoc test). (B) Survival curves. Log-rank (Mantel-Cox) test did not reveal any significant difference among groups. (C) Percent of body weight change at 40 mg/kg of DHA-dFdC. Student's t-test did not reveal any difference). In A and C, data shown are mean ± S.D.

2.4.2 Effect of DHA-dFdC at its RD-MTD on organ weights and blood and serum parameters in healthy mice

DHA-dFdC was given (i.v.) to healthy mice at its RD-MTD and the weights of major organs were measured at the end of the study. As shown in Fig. 2.2, at RD-MTD, DHA-dFdC did not significantly affect the weight of any of the major organs tested. In addition, at RD-MTD, DHA-dFdC did not cause any significant change in the blood or serum parameters tested, as compared to mice in the untreated group (Table 2.1).

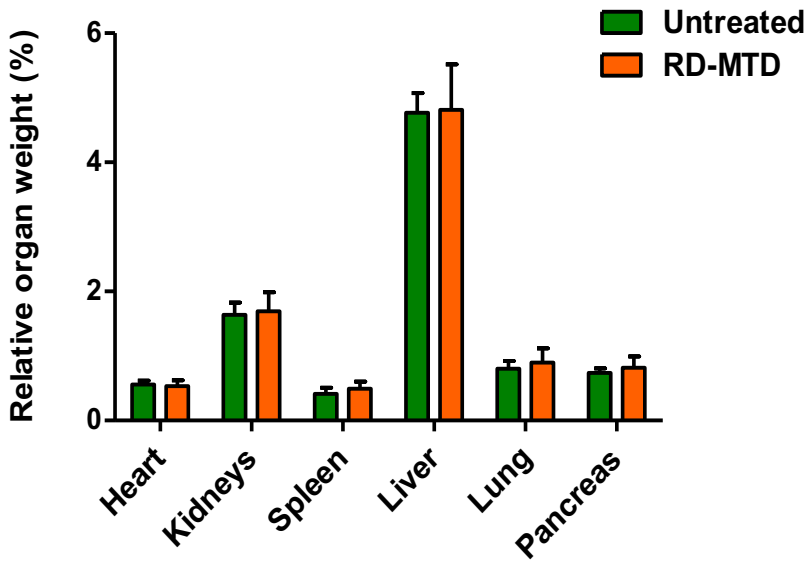


Fig. 2.2 Effect of DHA-dFdC at its RD-MTD on major mouse organ weights. Shown are relative organ weights (vs. body weights) 6 days after the completion of a schedule of five i.v. doses of DHA-dFdC at its RD-MTD. Data shown are mean ± S.D. (n = 8). Student's t-test did not reveal any significant different between the Untreated and RD-MTD groups.

Table 2.1 Blood and serum parameters in healthy mice 5 days after the last dose of DHA-dFdC at its RD-MTD. Data are mean ± S.D. (n = 6-8).

Parameter	Unit	RD-MTD (50 mg/kg)	
		Untreated	DHA-dFdC
Blood			
White blood cells (WBC)	k/μl	8.0 ± 3.5	5.4 ± 2.3
Red blood cell count (RBC)	M/ μl	10.1 ± 1.1	9.4 ± 1.0
Hematocrit (HCT)	%	40.0 ± 3.5	39.2 ± 4.2
Mean globular volume (MCV)	fL	39.8 ± 1.9	41.8 ± 1.7
Mean corpuscular hemoglobin (MCH)	pg	14.0 ± 0.3	14.5 ± 1.0
Mean corpuscular hemoglobin concentration (MCHC)	g/dL	35.3 ± 2.1	34.8 ± 2.7
Lymphocytes	%	79.9 ± 9.6	84.4 ± 7.8
Monocytes	%	5.5 ± 2.6	7.0 ± 3.9
Basophil	%	0.6 ± 1.0	0.2 ± 0.4
Eosinophil	%	2.9 ± 2.4	1.7 ± 1.6
Neutrophil Segmented	%	11.1 ± 7.6	6.8 ± 6.9
Platelet estimate		Adequate	Adequate
Serum			
Cholesterol	mg/dL	116.7 ± 17.0	127.6 ± 23.3
Glucose	mg/dL	262.5 ± 70.9	270.8 ± 39.7
Calcium	mg/dL	9.4 ± 2.2	7.0 ± 5.6
Phosphorus	mg/dL	14.6 ± 3.3	16.4 ± 1.5
Blood urea nitrogen (BUN)	mg/dL	23.5 ± 4.0	23.4 ± 1.7

Student's t-test did not reveal any significant different between Untreated and DHA-dFdC groups

Table 2.1 (continued)

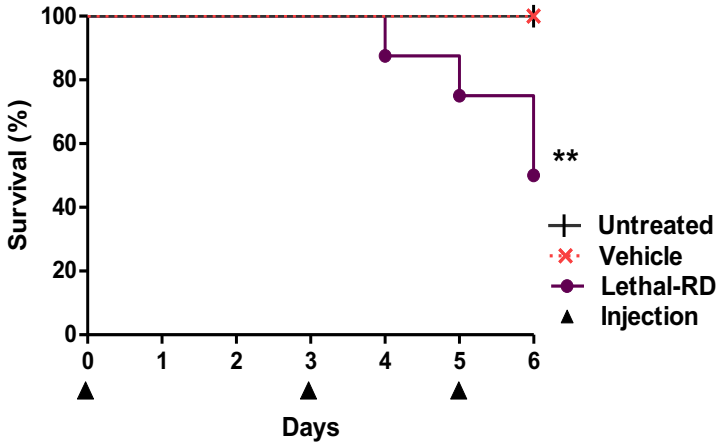
Globulin	g/dL	2.8 ± 0.5	2.7 ± 0.2
Total bilirubin	mg/dL	0.3 ± 0.1	0.2 ± 0.04
Total proteins	g/dL	6.0 ± 0.6	6.0 ± 0.2
Aspartate transaminase (AST)	U/L	689.5 ± 599.5	1266.0 ± 808.9
Alanine transaminase (ALT)	U/L	116.7 ± 115.2	154.2 ± 115.2
Alkaline phosphatase (ALP)	U/L	158 ± 39.4	145.0 ± 66.3
Albumin	g/dL	3.2 ±0.3	3.3 ± 0.2

Student's t-test did not reveal any significant different between Untreated and DHA-dFdC groups

2.4.3 Effect of a lethal-RD of DHA-dFdC on healthy mice

In order to identify a dose of DHA-dFdC that is lethal to DBA/2 mice, healthy mice were i.v. injected with three doses of DHA-dFdC at 100 mg/kg (i.e. on days 0, 3, and 5). Following the second dose, physical signs of toxicity observed include marked piloerection, transient paralysis, and little peer interactions. One day after the third dose, 50% of mice reached the end point (i.e. mortality or body weight loss of ≥ 20%) (Figs. 2.3A and 2.3B). Therefore, 100 mg/kg in a spaced schedule is considered a lethal-RD of DHA-dFdC in DBA/2 mice.

(A)



(B)

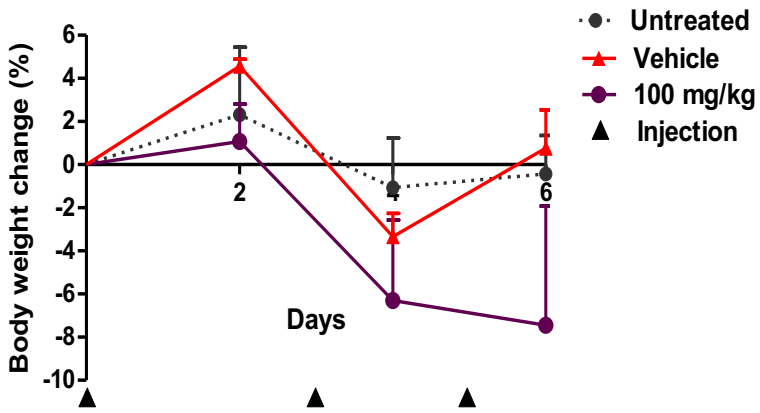


Fig. 2.3 Effect of a lethal-RD of DHA-dFdC following three i.v. injections (on days 0, 3, and 5 (▲)) on healthy DBA/2 mice (n = 8, half male, half female). (A) Survival curves (** $p < 0.01$, Lethal-RD vs. Untreated, Log-rank (Mantel-Cox) test). (B) Percent of body weight change. Data are mean ± S.D.

To identify the effect of a lethal-RD of DHA-dFdC on the main organs of mice, DHA-dFdC was i.v. injected to healthy mice at 100 mg/kg on days 0, 3, and 5, and the weight of major organs were measured at the end of the study (i.e. day 6). As shown in Fig. 2.4, the relative weights of the spleen and liver in mice that received the DHA-dFdC at the lethal-RD were significantly

lower than that of the untreated mice, whereas the vehicle in which the DHA-dFdC was dissolved in did not significantly affect the weights of any of the organs. At the lethal-RD, DHA-dFdC significantly affected some blood parameters, including the white blood cells (WBC), red blood cell count (RBC), hemoglobin level (HGB), hematocrit value (HCT), lymphocytes, eosinophil, and neutrophil segmented (i.e. neutrophil segs), as compared to mice in the untreated group (Table 2.2). In addition, the levels of some serum parameters such as phosphorus, blood urea nitrogen (BUN), and creatine phosphokinase (CPK) decreased in the lethal-RD group, as compared with the untreated group (Table II). Albumin also decreased in the lethal-RD group as compared with the untreated group, but was not different from that in the vehicle group (Table 2.2).

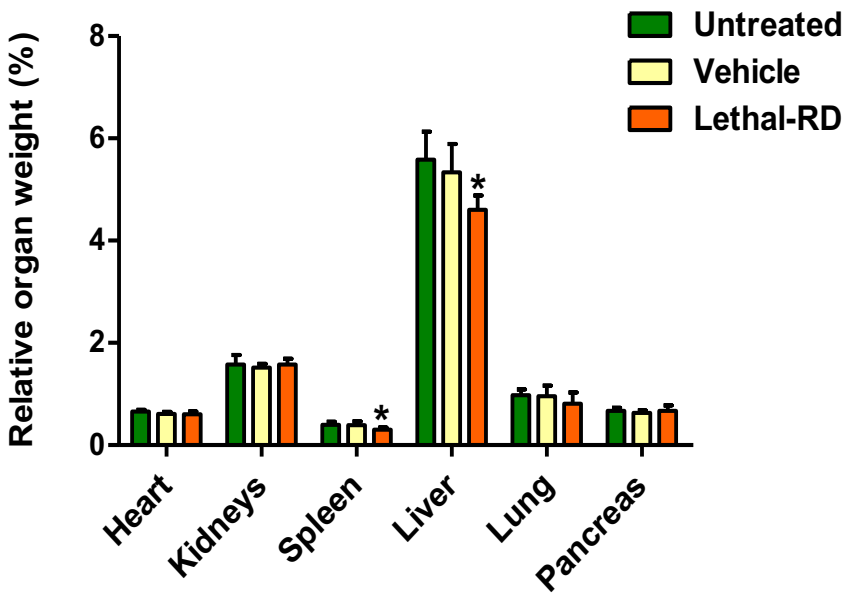


Fig. 2.4 Effect of DHA-dFdC at a lethal-RD on major organ weights of healthy mice. Shown are relative organ weights one day after the completion of three i.v. doses of DHA-dFdC at 100 mg/kg. Data shown are mean ± S.D. (n = 6-8) (* $p < 0.05$, Lethal-RD vs. Untreated, one-way ANOVA followed by Bonferroni post hoc test).

Table 2.2 Blood and serum parameters in healthy mice one day after three i.v. doses of DHA-dFdC at a lethal-RD (100 mg/kg). Data are mean ± S.D. (n = 8).

Parameter	Unit	Treatment		
		Untreated	Vehicle	DHA-dFdC
Blood				
White blood cells (WBC)	k/μl	5.2 ± 1.7	5.3 ± 2.1	3.2 ±1.4[a, b]
Red blood cell count (RBC)	M/ μl	9.7 ± 0.5	9.2 ± 0.7	7.9 ± 2.0[a]
Hemoglobin (HGB)	(g/dL)	13.8 ± 0.9	13.8 ± 1.1	11.7 ± 2.5[a, b]
Hematocrit (HCT)	%	39.8 ± 3.2	38.2 ± 2.1	32.0 ± 7.5[a, b]
Mean globular volume (MCV)	fL	41.0 ± 3.8	41.4 ± 1.8	41.0 ± 2.0
Mean hemoglobin quantity (MCH)	pg	14.3 ± 1.1	14.9 ± 0.5	15.1 ± 1.2
Mean cellular hemoglobin concentration (MCHC)	g/dL	35.0 ± 4.1	36.2 ± 2.3	36.9 ± 2.5
Lymphocytes	%	69.9 ± 9.7	75.1 ± 14.4	84.7 ± 6.2[a]
Monocytes	%	9.2 ± 9.0	6.7 ± 2.0	8.5 ± 2.9
Basophil	%	1.0 ± 1.5	2.1 ± 2.6	1.4 ± 2.9

Statistical analysis was performed using one-way ANOVA followed by a Bonferroni post hoc test.

[a] $p < 0.05$ vs. Untreated; [b] $p < 0.05$ vs. Vehicle; ∞ 7 adequate, 1 decreased.

Table 2.2 (continued)

Eosinophil	%	3.8 ± 3.0	2.8 ± 1.6	0.8 ± 1.2 [a, b]
Neutrophil Segmented	%	16.1 ± 5.6	13.4 ± 12.3	4.6 ± 3.6 [a]
Platelet estimate		Adequate	Adequate	Adequate
Serum				
Cholesterol	mg/dL	103.7 ± 22.3	99.7 ± 9.2	94.6 ± 17.5
Glucose	mg/dL	230.4 ± 44.0	199.7 ± 37.5	210.1 ± 45.4
Calcium	mg/dL	9.9 ± 1.7	8.8 ± 3.4	9.9 ± 0.8
Phosphorus	mg/dL	14.3 ± 1.7	13.8 ± 1.5	11.9 ± 1.7a
Bicarbonate	(mmol/L)	28.9 ± 2.7	33.3 ± 2.8	31.6 ± 4.8
Blood urea nitrogen (BUN)	mg/dL	22.1 ± 3.0	20.5 ± 2.2	17.9 ± 1.96 [a, b]
Globulin	g/dL	2.7 ± 0.1	2.7 ± 0.2	2.7 ± 0.1
Total bilirubin	mg/dL	0.3 ± 0.1	0.4 ± 0.2	0.3 ± 0.1
Direct bilirubin	mg/dL	0.1 ± 0.04	0.1 ± 0.05	0.1 ± 0.05
Indirect bilirubin	mg/dL	0.2 ± 0.08	0.3 ± 0.1	0.2 ± 0.08

Statistical analysis was performed using one-way ANOVA followed by a Bonferroni post hoc test.
[a] $p < 0.05$ vs. Untreated; [b] $p < 0.05$ vs. Vehicle; ∞ 7 adequate, 1 decreased.

Table 2.2 (continued)

Aspartate transaminase (AST)	U/L	200.6 ± 91.6	279.9 ± 165.3	193.9 ± 69.3
Alanine transaminase (ALT)	U/L	21.3 ± 3.0	25.9 ± 8.0	18.8 ± 5.1
Alkaline phosphatase (ALP)	U/L	200.3 ± 29.1	161.9 ± 70.2	182.6 ± 21.6
Creatine phosphokinase (CPK)	U/L	1033.7 ± 609.6	1923.3 ± 1248.2	822.4 ± 451.6[b]
Albumin	g/dL	2.8 ± 0.1	2.5 ± 0.3[a]	2.4 ± 0.2[a]

Statistical analysis was performed using one-way ANOVA followed by a Bonferroni post hoc test.

[a] $p < 0.05$ vs. Untreated; [b] $p < 0.05$ vs. Vehicle; ∞ 7 adequate, 1 decreased.

2.4.4 Efficacy of DHA-dFdC at or below its RD-MTD *in vivo*

The RD-MTD identified above was in DBA/2 mice, and therefore DBA/2 mice were i.p injected (on day 0) with the syngeneic L1210 leukemia cells to establish a mouse model of leukemia to evaluate the antitumor activity of DHA-dFdC at or below its RD-MTD. Mice were inoculated on day 0, and then received (i.v.) five doses of DHA-dFdC on days 1, 4, 8, 11, and 14. Mice in the control groups (untreated mice and vehicle alone) were sacrificed on day 18, because they reached our end point (i.e. body weight increase of ≥ 60%) (Figs. 2.5A and 2.5B). Those mice presented larger distended abdomens, urine staining, and thin body conditions. In contrast, mice treated with DHA-dFdC at or below its RD-MTD did not gain more than 10% of body weight (Fig. 2.5B), and they appeared normal on day 18. Mice that were treated with DHA-dFdC at or below its RD-MTD survived longer than mice in the control groups (Fig. 2.5A and Table 2.3). DHA-dFdC at its RD-MTD (50 mg/kg) showed a higher T/U ratio than at 3/4 x RD-MTD (37.5 mg/kg) or at 1/2 x RD-MTD (25 mg/kg) (Table 2.3). Furthermore, DHA-dFdC at its RD-MTD increased mouse life span by 44.4%, 27.0% at 3/4 x RD-MTD, and 21.4% at 1/2 x RD-MTD (Table 2.3).

(A)

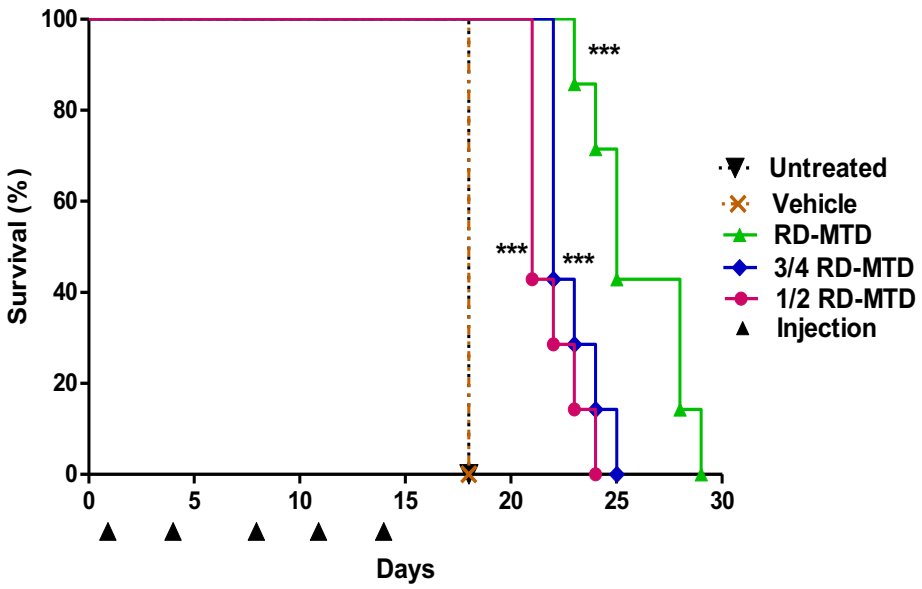


Figure 2.5 continued next page

(B)

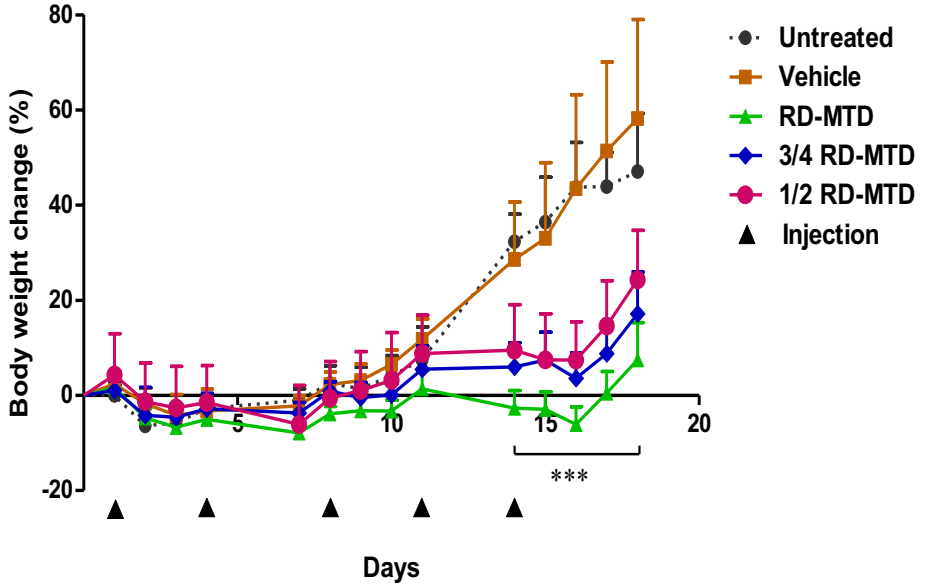


Fig. 2.5 Efficacy of DHA-dFdC against L1210 leukemia cells in a mouse model. Tumor cells were injected (i.p.) on day 0. On days 1, 4, 8, 11, and 14 (▲), mice (n = 7) were i.v. injected with DHA-dFdC at 1 x RD-MTD, ¾ x RD-MTD, or ½ x RD-MTD. Mice in control groups were i.v. injected with vehicle alone or left untreated. (A) Survival curves (*** $p < 0.0001$, all DHA-dFdC groups vs. Untreated, Log-rank (Mantel-Cox) test). (B) Percent of body weight change. Data shown are mean ± S.D. (*** $p < 0.0001$, all DHA-dFdC groups vs. Untreated, one-way ANOVA followed by Bonferroni post hoc test).

Table 2.3 Antitumor activity of DHA-dFdC at 1 x RD-MTD, ¾ x RD-MTD, and ½ x RD-MTD in mice with syngeneic L1210 leukemia cells. Data are mean ± S.D. (n = 7).

Groups	Dose (mg/kg)	Days of treatment	Survival (day)	T/U*	% ILS+
Untreated	-	-	18	1.0	0
Vehicle	0	1, 4, 8, 11, 14	18	1.0	0
1 x RD-MTD	50	1, 4, 8, 11, 14	26.0 ± 2.3[a, b]	1.4 ± 0.13[a, b]	44.4
3/4 x RD-MTD	37.5	1, 4, 8, 11, 14	22.9 ± 1.2[a, b]	1.3 ± 0.07[a, b]	27.0
1/2 x RD-MTD	25	1, 4, 8, 11, 14	21.9 ± 1.2[a, b]	1.2 ± 0.07[a, b]	21.4

* The ratio of the mean survival time of treated group to the median survival time of the untreated group.
+ Increase in life span compared to untreated group.
Statistical analysis was performed using one-way ANOVA followed by a Bonferroni post hoc test.

[a] $p < 0.0001$ vs. Control groups (i.e. Untreated and Vehicle);

[b] $p < 0.0001$ vs. 50 mg/kg.

2.5 DISCUSSION

The repeat-dose range finding study showed that DHA-dFdC has a dose-dependent toxicity in terms of body weight and survival (i.e. weight loss of > 20%). Its RD-MTD is 50 mg/kg (Figs. 2.1 - 2.2 and Table 2.1). At or below its RD-MTD, DHA-dFdC is effective in prolonging mouse survival in a mouse leukemia model (Fig. 2.5). However, higher doses of DHA-dFdC following a repeat-dose schedule affected the survival and body weight of the mice significantly (Figs. 2.1A and 2.1B).

A single i.v. dose of DHA-dFdC at 100 mg/kg was not lethal to mice (data not shown). Due to the limited solubility of DHA-dFdC in the vehicle solution, we tested whether multiple

doses of DHA-dFdC at 100 mg/kg in a spaced schedule is lethal to mice. Mice were i.v. injected with DHA-dFdC at 100 mg/kg three times on days 0, 3, and 5. At this dosing regimen, around 50% of mice reached the end point (i.e. ≥ 20% of body weight loss) as shown Figs. 2.3A and 2.3B. In addition, the spleen and liver weights of those mice decreased significantly in comparison to mice in the control groups (Fig. 2.4A). Furthermore, at this lethal-RD, DHA-dFdC showed hematological toxicity, because certain blood parameters (e.g. WBC, RBC, HGB, HCT, lymphocytes, eosinophil, and neutrophil segmented) were significantly different from that of mice in the untreated group (Table 2.2). Gemcitabine has dose-limiting toxicities in clinics. For example, data from clinical studies showed that gemcitabine causes dose-limiting myelosuppression that is characterized mainly by thrombocytopenia (1, 8). In our study, at a lethal-RD dose, DHA-dFdC significantly decreased the levels of WBC as well as RBC, when compared to the control groups (40% and 18%, respectively) (Table 2.2). On the other hand, our data showed that platelet estimate was adequate, indicating that DHA-dFdC may not cause significant thrombocytopenia. At the lethal-RD used, DHA-dFdC also induced a significant decrease in the levels of HGB and HCT (15% and 20%, respectively), indicating DHA-dFdC induces anemia in DBA/2 mice (38, 39). The neutrophil levels in mice that received the lethal-RD of DHA-dFdC decreased significantly as compared to the untreated group (71%), indicating an acute neutropenia. It was reported that the number of WBC decreased in healthy men when they received a diet supplemented with DHA (22). The main cause of reduction of WBC number was attributed to a reduction in the number of circulating neutrophils (22). In our study, we found that at lethal-RD, DHA-dFdC caused a decrease in the number of WBC as well as neutrophils segmented (Table 2.2). Both the DHA and gemcitabine moieties in the DHA-dFdC may have contributed to the observed hematological toxicity. Some serum parameters such as phosphorus, BUN, CPK and albumin in mice that received DHA-dFdC at the lethal-RD were also decreased compared to the untreated group (Table 2.2), but these changes were not more than 20%. According a previous study (40), small changes in some parameters in toxicity studies are due to animals having a poor tolerance to the test article. Indeed, one of the most common effects of toxicity studies in rodents

is decreasing serum albumin concentration because small species have a rapid turnover of albumin (41). Finally, DHA-dFdC at the lethal-RD did not significantly affect serum levels of aspartate transaminase (AST), alanine transaminase (ALT), and alkaline phosphatase (ALP), when compared to the untreated control, in spite of the finding that it caused a decrease in the relative weight of mouse liver (Fig. 2.4). Data from a previous phase II clinical study showed that the liver toxicity of gemcitabine was mild and transient (1).

DHA is considered a generally recognized as safe material for inclusion in diet by the U.S. Food and Drug Administration (42). However, some side effects have been reported such as gastrointestinal disturbances, nausea, increased bleeding time, effects in the glycemic control in non-insulin-dependent diabetics, and increased levels of low-density lipoprotein cholesterol (42). Parenteral omega-3 fatty acid preparations have been used for years as part of total parenteral nutrition and are generally well tolerated (27, 43). In addition, an *in vitro* study reported that pancreatic stellate cells (PSCs) are highly sensitive to gemcitabine plus Lipidem® (i.e. emulsion for infusion that contains omega-3-acid triglyceride) (44). As PSCs are responsible of therapeutic resistance of pancreatic cancer due to their contribution to secretion of extracellular matrix, the anti-proliferative and anti-invasive efficacy of gemcitabine plus Lipidem® treatment reported in PSC culture might be a good alternative to targeting pancreatic cancer.

2.6 CONCLUSION

The RD-MTD of DHA-dFdC in a spaced schedule is 50 mg/kg when intravenously injected to DBA/2 mice. DHA-dFdC mainly affects mouse spleen at a lethal repeat dose. At or below its RD-MTD, DHA-dFdC shows anticancer activity in a mouse model of leukemia, improving the survival of leukemia-bearing mice.

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Chapter 3: A Solid Lipid Nanoparticle Formulation of 4-(*N*)-Docosahexaenoyl 2′, 2′-Difluorodeoxycytidine, a Compound with Potent, Broad Spectrum Antitumor Activity

3.1 ABSTRACT

Previously, we reported 4-(*N*)-docosahexaenoyl 2′, 2′-difluorodeoxycytidine (DHA-dFdC), a novel lipophilic compound with a potent, broad spectrum antitumor activity. Herein, we report a solid lipid nanoparticle (SLN) formulation of DHA-dFdC with improved apparent aqueous solubility and chemical stability. The SLNs were prepared from lecithin/glycerol monosterate-in-water emulsions emulsified with D-α-tocopherol polyethylene glycol 1000 succinate (TPGS) and Tween 20. The resultant DHA-dFdC-SLNs were 102.2 ± 7.3 nm in diameter and increased the solubility of DHA-dFdC in water to at least 5.2 mg/mL, more than 200-fold higher than its intrinsic water solubility. As a comparison, the waxy solid of DHA-dFdC, even in the presence of vitamin E as an antioxidant, was unstable when stored at room temperature. However, after one-month of storage at the same condition, DHA-dFdC in lyophilized DHA-dFdC-SLNs powder did not significantly degrade. DHA-dFdC-SLNs also showed an increased cytotoxicity against certain tumor cells than DHA-dFdC. The plasma concentration of DHA-dFdC in mice intravenously injected with DHA-dFdC-SLNs in dispersion followed a bi-exponential model, with a half-life of ~44 h. In mice with pre-established B16-F10 murine melanoma, DHA-dFdC-SLNs were significantly more effective than DHA-dFdC in controlling the tumor growth. In addition, histology evaluation revealed a high level of apoptosis and tumor encapsulation in tumors in mice treated with DHA-dFdC-SLNs. DHA-dFdC-SLNs represents a new DHA-dFdC formulation with improved antitumor activity.

3.2 INTRODUCTION

Gemcitabine (2´, 2- difluorodeoxycytidine, dFdC) is a nucleoside analogue approved for treatment of pancreatic, lung, breast, and ovarian cancer by slow intravenous infusion (1-4). In an effort to improve the efficacy of gemcitabine, we previously synthesized a new compound DHA-dFdC by conjugating docosahexaenoic acid (DHA), a omega-3 polyunsaturated fatty acid (PUFA), to dFdC on the 4-*N* position (5). DHA-dFdC showed potent and broad spectrum antitumor activity against NCI-60 DTP human tumor cell lines and was significantly more effective than the molar equivalent dose of gemcitabine in controlling pancreatic tumor growth in several mouse models of pancreatic cancer, including a genetically engineered mouse model and athymic mice with orthotopically implanted human pancreatic tumor cells (5). The repeat dose-maximum tolerated dose of DHA-dFdC in a Tween 80-ethanol in water solution was found to be 50 mg/kg in C57BL/6 mice (6). However, DHA-dFdC is poorly soluble in water (i.e. intrinsic solubility, ~25 μg/mL) and is not chemically stable in the current Tween 80-ethanol in water formulation (5).

SLNs have emerged as an attractive delivery system for poorly water-soluble drugs (7-9). There is also evidence that incorporation of drug into SLNs can increase its chemical stability (10-12). Previously, our group developed a SLN formulation based on a lecithin/glycerol monostearate (GMS)-in-water emulsion (13-15). In the present study, to improve the (apparent) water solubility and the chemical stability of DHA-dFdC, we developed a new DHA-dFdC SLN formulation (DHA-dFdC-SLNs) by incorporating DHA-dFdC into SLNs prepared by emulsifying lecithin/GMS-in-water emulsions with Tween 20 and TPGS (or vitamin E TPGS) (13, 14). TPGS is a water-soluble derivate of natural vitamin E, which is formed by esterification of vitamin E succinate with polyethylene glycol (PEG) (16). TPGS is widely used in pharmaceutical formulations as an emulsifier, solubilizer, absorption enhancer, permeation enhancer, and/or stabilizer (16-19). There is also evidence that TPGS has a stronger antioxidant activity than α-tocopherol or vitamin E (20, 21). Moreover, TPGS is a P-gp inhibitor and can help overcome multidrug resistance by tumor cells (16, 19, 22, 23). Furthermore, data from several studies showed that TPGS induces apoptosis and has a synergic effect with certain cancer chemotherapeutic agents

such as docetaxel, paclitaxel, and doxorubicin (23-27). Finally, we also evaluated the plasma pharmacokinetics of DHA-dFdC in the DHA-dFdC-SLNs in a mouse model, the cytotoxicity of the DHA-dFdC-SLNs against several tumor cell lines *in vitro*, and the antitumor activity of the DHA-dFdC-SLNs in a mouse model with B16-F10 murine melanoma cell.

3.3 MATERIALS AND METHODS

3.3.1 Materials and cell lines

Mannitol, Tween 20, GMS, TPGS, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Tween 80, mannitol, and sucrose were from Sigma-Aldrich (St. Louis, MO). Gemcitabine HCl was from Biotang, Inc. (Lexington, MA). Soy lecithin was from Alfa Aesar (Ward Hill, MA). Ethyl acetate (EtOAc), dimethyl sulfoxide, tetrahydrofuran (HPLC-grade), isopropanol, and methanol (HPLC-grade) were from Thermo Fisher (Waltham, MA). Float-A-Lyzer®G2 dialysis device (MWC 50 kD) was from Spectrum Inc. (New Brunswick, NJ)

B16-F10 murine melanoma cell and TC-1 murine lung cancer cell lines were from the American Type Culture Collection (Manassas, VA). M-Wnt cells (murine mammary gland cell lines) were from Dr. Stephen D. Hursting's lab at The University of North Carolina, Chapel Hill. B16-F10 and TC-1 cells were grown in DMEM and RPMI 1640, respectively (Invitrogen, Carlsbad, CA). M-Wnt cells were grown in a similar medium as TC-1, with an additional supplement of 1% Glutamax (GlutaMAX™Supplement, Gibco®). All media were supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/mL of penicillin, and 100 μg/mL of streptomycin, all from Invitrogen (Carlsbad, CA).

3.3.2 Preparation and characterization of 4-(*N*)-docosahexaenoyl 2′,2′-difluorodeoxycytidine solid lipid nanoparticles (DHA-dFdC-SLNs)

3.3.2.1 Preparation of DHA-dFdC-SLNs

DHA-dFdC was synthesized following our previously reported conjugation scheme (5). The purity of the resultant DHA-dFdC was confirmed by NMR and Mass Spectrum. SLNs were prepared as previously described with some modifications (14). Briefly, 3.5 mg of soy lecithin, 0.5 mg of GMS, and 0.875 mg of TPGS were weighed into a 7 mL glass vial. Eight hundred microliter of de-ionized and filtered (0.22 μm) water (80°C) was added into the lecithin/GMS/TPGS mixture,

which was then vortexed and sonicated for 3 min until a homogenous slurry was formed. The mixture was maintained on an 80°C hot plate surface for 5 min. A solution of Tween 20 (55 mg in 1 ml of water) was prepared, and 200 μL of this solution was added drop wise into the mixture to reach a final concentration of 1% (v/v). The resultant emulsions were allowed to cool to room temperature while stirring to form SLNs. To incorporate DHA-dFdC into the SLNs, DHA-dFdC at various amounts (i.e. 5.2, 8.3, 9.8, or 14.3 mg) were added into the lecithin/GMS/TPGS mixture before the addition of water. The next steps were identical to the preparation of DHA-dFdC-free SLNs. The size and zeta potential of the SLNs were measured using a Malvern Zetasizer Nano ZS (Westborough, MA)

3.3.2.2 Short-term stability study

The stability of DHA-dFdC-SLNs prepared with 0, 5.2, 8.3, 9.8, or 14.3 mg of DHA-dFdC was evaluated at 4°C for 6 days. The size and zeta potential of the SLNs were measured using a Malvern Zetasizer Nano ZS (Westborough, MA).

3.3.2.3 Transmission electron microscopy (TEM)

The size and morphology of the DHA-dFdC-SLNs were examined using a transmission electron microscope available in the Institute for Cellular and Molecular Biology Microscope and Imaging Facility at The University of Texas at Austin. The carbon film-coated copper grid was glow discharged for 2 min. A sample of 10 μL of DHA-dFdC-SLNs suspended in water was deposited on the grid and left to stand for 1 min. The excess sample was removed with a filter paper. One drop of 1% uranyl acetate was added on the grid for 30 s. The sample was then observed under the TEM after removing the excess uranyl acetate fluid with filter paper (28).

3.3.2.4 Encapsulation efficiency (EE)

The encapsulation efficiency of DHA-dFdC in SLNs was determined by an ultrafiltration method. Briefly, 1 mL of DHA-dFdC-SLNs was added into an ultrafiltration centrifuge tube (30 kD, Amicon Ultra-4, Millipore) and centrifuged at 2844 rcf for 10 min. Then, 100 μl of the filtrate solution was taken from the bottom part of the ultrafiltration centrifuge tube to measure DHA-dFdC concentration by high performance liquid chromatography (HPLC). To corroborate the detection method, the remaining suspension (≈50 μl) in the ultrafiltration centrifuge tube was re-dissolved with 950 μl water to extract the DHA-dFdC, according the procedure previously describe.

3.3.2.5 Gel permeation chromatography (GPC)

To separate potential micelles from DHA-dFdC-SLNs, GPC was performed using a 6 mm x 30 cm Sepharose® 4B column (13). Samples (100 μL) were applied into the column and eluted with de-ionized and filtered (0.22 μm) water. Elution fractions of 500 μL were collected. Particle concentration (i.e. kilo counts per second or Kcps) in each fraction was measured using a Malvern Zetasizer Nano ZS, and the concentration of DHA-dFdC in each fraction was determined using HPLC after extraction.

3.3.3 Lyophilization of the DHF-dFdC-SLNs and their stability in lyophilized powder

A 30% (w/v) stock solution of sucrose as lyoprotectant was prepared with de-ionized and filtered (0.2 μm) water. Then, 900 μL of DHA-dFdC-SLNs in water suspension was mixed with 100 μL of the sucrose solution to obtain a final suspension with 3% (w/v) of sucrose. The DHA-dFdC-SLNs in suspension were then stored at -20°C for 30 min, transferred to -80°C for 60 min, and finally transferred to a VirTis Advantage bench top tray lyophilizer (The VirTis Company, Inc. Gardiner, NY). Lyophilization was performed over 72 h at pressure less than 200 mTorr under nitrogen atmosphere. The shelf temperature was gradually ramped from -40°C to 26°C. After

lyophilization, the sample was quickly sealed and stored in a desiccator at room temperature, protected from light. To evaluate the physical and chemical stability of the DHA-dFdC-SLNs in the lyophilized powder, DHA-dFdC was extracted from the powder 0, 7, and 30 days later. Briefly, the lyophilized sample was reconstituted in 1 mL of de-ionized and filtered (0.2 μm) water. The reconstituted DHA-dFdC-SLN suspension (100 μL) was mixed with 100 μL of isopropanol, vortexed for 30 s, and maintained at room temperature for 5 min. Then, 600 μl of ethyl acetate was added, and the sample was vortexed for 30 s and centrifuged at 11,000 rcf for 20 min. The supernatant was collected into a glass vial and evaporated under nitrogen. The sample was re-dissolved in 100 μL of THF, and the concentration was measured by HPLC.

As a control, DHA-dFdC was dissolved in ethanol and mixed with vitamin E at final concentration of 5.047% (w/w) (5). The solution was dried under nitrogen, sealed, and stored at room temperature, protected from light, and the content of DHA-dFdC was measured at various time points

3.3.4 *In vitro* stability of DHA-dFdC-SLNs in simulated biological media

To evaluate the stability of the DHA-dFdC-SLNs in simulated biological media, the SLNs in suspension were diluted in phosphate-buffered saline (PBS, 10 mM, pH 7.4) with 10% FBS (v/v) and incubated at 37°C in a MaxQ 4000 Floor Shaker Incubator (Thermo Fisher Scientific, 100 rpm) for 18 h. The particle size was measured at different time points using a Malvern Zetasizer.

3.3.5 *In vitro* release of DHA-dFdC from DHA-dFdC-SLNs

The release profile of DHA-dFdC from SLNs were evaluated by suspending DHA-dFdC-SLNs at concentration of 127 μg/mL in release medium (1% (w/v) of Tween 20 dissolve in PBS), which were then placed into a 1 mL cellulose ester dialysis tube (MWC 50 kD) from Spectrum

Chemicals & Laboratory Products (New Brunswick, NJ). The dialysis tube was then placed into a plastic conical tube containing 13 mL of release medium to created sink conditions, which was incubated in a MaxQ 5000 Floor Shaker Incubator at 37°C and 100 rpm for 8 h. At predetermined time points, 200 μL of the release medium was withdrawn and replaced with 200 μL of fresh release medium. As a control, the diffusion of DHA-dFdC dissolved in a Tween 20 solution (i.e. 127 μL g/mL of DHA-dFdC in 1% of Tween 20 in water) across the dialysis membrane was also measured. The concentration of the DHA-dFdC was determined by HPLC.

3.3.6 HPLC

HPLC analysis of DHA-dFdC was performed using an Agilent Infinity 1260 (Santa Clara, CA) with a RP-C18 column (Zorbax Eclipse, 5 μm, 4.5 mm × 150 mm, Santa Clara, CA). The mobile phase was methanol and water (90:10, v/v). The flow rate was 1.0 ml/min, and the detection wavelength and injection volume were 248 nm and 5 μL, respectively (5).

3.3.7 *In vitro* cytotoxicity assay

The cytotoxicity of DHA-dFdC-SLNs was evaluated in TC-1, B16-F10, and M-Wnt cells. Cells were seeded into 96-well plates (4000 cells/well for TC-1 and B16-F10 cells, 1000 cells/well for M-Wnt cells) and incubated at 37°C, 5% CO2 overnight. Cells were then treated with various concentrations of DHA-dFdC, DHA-dFdC-SLNs, DHA-dFdC-free SLNs, or dimethyl sulfoxide for up to 48 h. As a control, cells were treated with fresh medium. Cell survival was determined using an MTT assay (29). DHA-dFdC was dissolved in dimethyl sulfoxide and then diluted with cell culture media, whereas DHA-dFdC-SLNs and DHA-dFdC-free SLNs were dispersed directly in cell culture media.

3.3.8 Plasma pharmacokinetics (PK) of DHA-dFdC in DHA-dFdC-SLNs

The animal protocol was approved by the Institutional Animal Care and Use Committee at The University of Texas at Austin. To evaluate the PK parameters, healthy female C57BL/6 mice (6-8 weeks, Charles River Laboratories, Wilmington, MA) were injected intravenously with DHA-dFdC-SLNs dispersed in sterile mannitol 5% (w/v) at dose of 2 mg of DHA-dFdC per mouse. Mice were euthanized at various time points later (i.e. 0.25, 0.5, 1, 2, 4, 8, 24, and 48 h). Blood was collected into heparin-coated tubes, which were then centrifuged at 13000 rcf for 20 min to isolate plasma. Then 200 μL of the plasma was mixed with 200 μL of isopropanol and 200 μL cold PBS. The mixture was vortexed and incubated at 4°C for 5 min. Following the incubation, 1000 μL of ethyl acetate was added, and the mixture was vortexed for 5 min, and followed by centrifugation at 18,000 rcf for 5 min. The supernatant was collected and dried under nitrogen gas. Finally, the residue was re-dissolved in 100 μL of THF, which was then analyzed using HPLC (5). As an internal control, 4-(*N*)-stearoyl dFdC synthesized by conjugating stearate and dFdC on its 4-(*N*) position was added in the samples before extraction (13). Data were analyzed using PK Solver®, assuming a two-compartmental model (30).

3.3.9 Evaluation of the antitumor activity of DHA-dFdC-SLNs in a mouse model

Female C57BL/6 mice (18-20 g, 6-8 weeks) were subcutaneously (s.c.) injected with B16-F10 (5×10^5 cells/mouse) in the right flank on day 0. Seven days later, mice were randomized in 5 groups (n = 5-6) and i.v. injected with DHA-dFdC (1 mg/mouse, equivalent to 50 mg/kg) dissolved a vehicle solution (i.e. Tween 80 (10%, w/v), ethanol (5.2%, v/v), and mannitol (5%, w/v) in water), the vehicle solution (as a control) (5, 6), DHA-dFdC-SLNs (equivalent to 1 mg of DHA-dFdC/mouse), or the equivalent dose of DHA-dFdC-free SLNs; both SLNs were dispersed in sterile mannitol 5%, (w/v). As a control, one group of mice were left untreated. Treatments were repeated every 3 days for a total of 4 times. Mouse health and tumor growth were monitored daily. Tumor size was measured 2-3 times a week, and tumor volume was calculated as: volume (mm^3)

= (length x width^2)/2. Mice were euthanized 17 days after B16-F10 cell injection, and tumor tissues were collected for histology study. For mice that were left untreated, the length of some of the tumors reached 15 mm before day 17 and had to be euthanized earlier.

3.3.10 Histology

Tumor tissues were fixed in formalin, embedded, and stained with hematoxylin and eosin (H&E) in the Histological and Tissue Analysis Facility in the Dell Pediatric Research Institute at The University of Texas at Austin.

3.3.11 Data analysis

Statistical analyses were completed by one-way ANOVA followed by a Bonferroni post hoc test. A p value of ≤ 0.05 (two-tail) was considered significant. Most of the analyses were performed with GraphPad Prism (GraphPad Software, Inc., La Jolla, CA). PK parameters were obtained using PK Solver (30).

3.4 RESULTS AND DISCUSSION

3.3.1 Preparation and characterization of DHA-dFdC-SLNs

DHA-dFdC is a lipophilic compound with potent antitumor activity against various cancer cell lines in culture (e.g. pancreatic cancer, leukemia, kidney cancer) and in mouse models of pancreatic cancer and leukemia (5, 6). However, this compound presents solubility and stability issues (5). To increase its water solubility and improve its chemical stability, we developed a solid lipid nanoparticle formulation (13, 14).

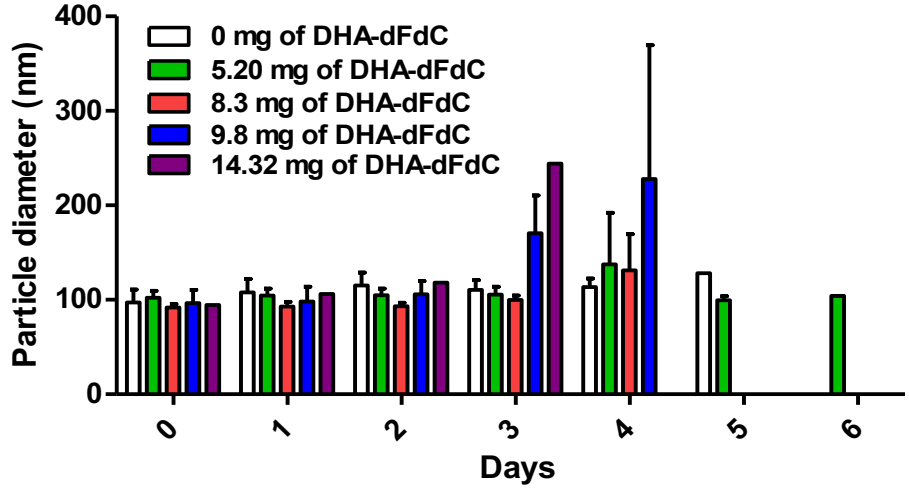
The particle diameter, polydispersity index, and zeta potential DHA-dFdC-SLNs loaded with various concentrations/amounts of DHA-dFdC are shown in Table 3.1. Statistical analysis did not reveal any significant differences on the particle sizes and zeta potentials of SLNs prepared with various amounts of DHA-dFdC. However, in a short stability study at 4°C, the DHA-dFdC-SLNs prepared with 5.2 mg of DHA-dFdC remained stable after 6 days (Fig.3.1A-C) and were thus selected for further studies. This SLN formulation increases the apparent aqueous solubility of DHA-dFdC to 5.2 mg/ml, which may be further increased by concentrating the nanoparticles. Shown in Fig. 1D is the dynamic light scattering spectrum of DHA-dFdC-SLNs prepared with 5.2 mg of DHA-dFdC. The TEM images of the DHA-dFdC-SLNs showed that they are spherical (Fig. 3.1E) with particle size smaller than that determined by dynamic light scattering (Fig. 3.1D). The encapsulation efficiency of DHA-dFdC in the DHA-dFdC-SLNs was close to 100% since DHA-dFdC was not detected in the filtrate after ultrafiltration. To corroborate this result, the suspension remained in the ultrafiltration centrifuge tube was re-dissolved in water to extract the DHA-dFdC, and 97% ± 21.4 (n = 6) of DHA-dFC was recovered. There are reports that TPGS as an emulsifier in paclitaxel-loaded polymeric nanoparticles helped to improve the encapsulation efficiency to 100% (16, 17, 31). Due to the presence of Tween 20 and TPGS in our DHA-dFdC-SLN formulation, it is possible that a certain fraction of the DHA-dFdC may be in micelles. For instance, TPGS has a relative low critical micelle concentration of 0.02 % (w/w) at 37ºC, ~ 1% (w/v) for

Tween 20 at 20ºC (32, 33). GPC was applied to identify the extent to which DHA-dFdC is potentially incorporated into micelles (13). As shown in Fig. 3.1F, only one apparent DHA-dFdC peak can be identified in the GPC spectrum, which also overlaps with particle count spectrum, providing additional evidence that almost all the DHA-dFdC was encapsulated into DHA-dFdC-SLNs.

Table 3.1 Characterization of DHA-dFdC-SLNs prepared with different amounts of DHA-dFdC. Data shown are mean ± S.D. (n = 3).

DHA-dFdC (mg)	0	5.2	8.3	9.8
Particle diameter (nm)	97.3 ± 13.6	102.2 ± 7.3	92.0 ± 3.6	96.5 ± 14.2
Polydispersity index	0.27 ± 0.10	0.23 ± 0.01	0.24 ± 0.02	0.26 ± 0.02
Zeta potential (mV)	-51.5 ± 0.1	-55.3 ± 3.0	-60.7 ± 2.4	-57.7 ± 2.9

(A)



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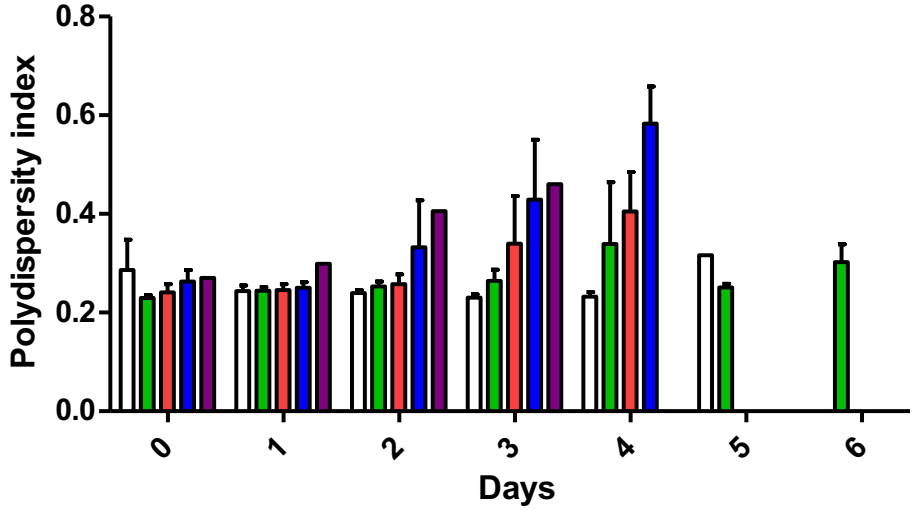

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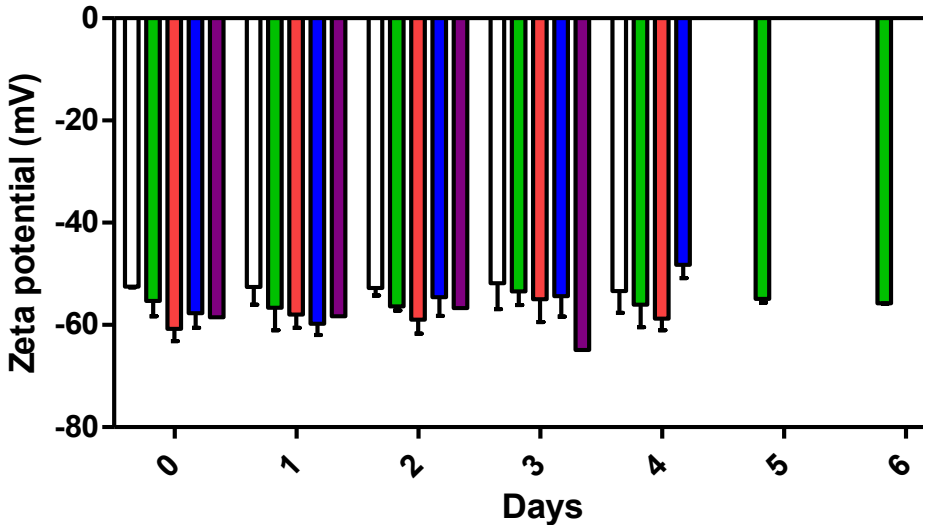

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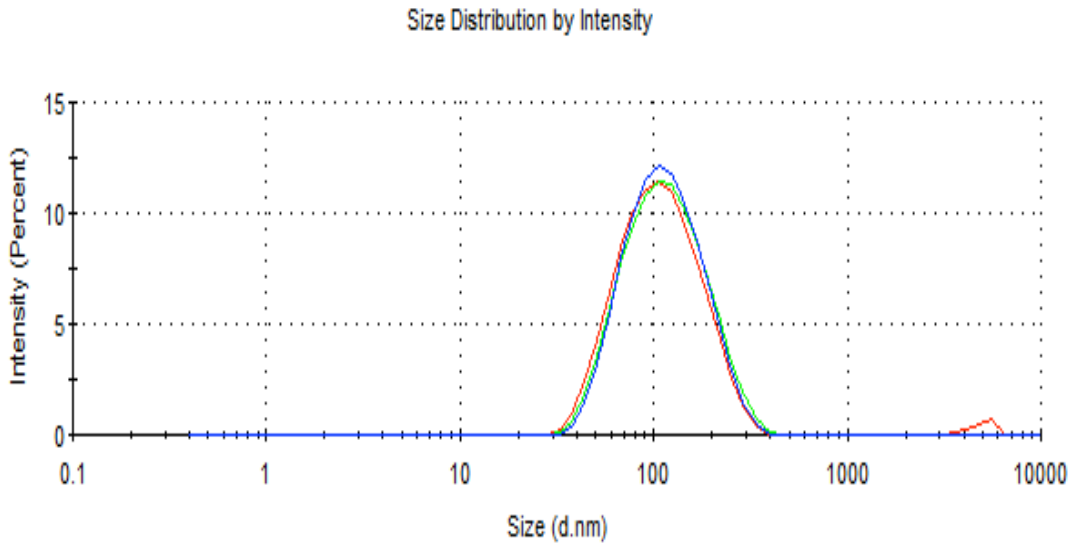
(D)



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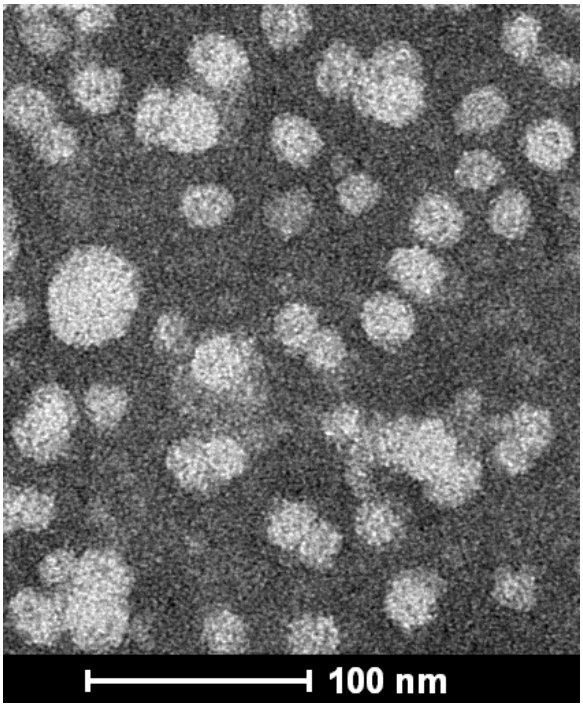

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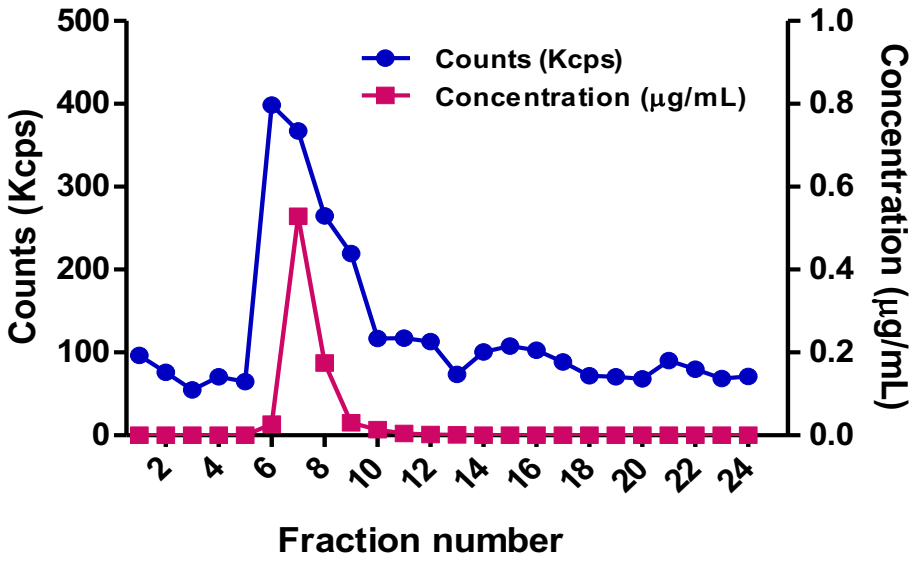
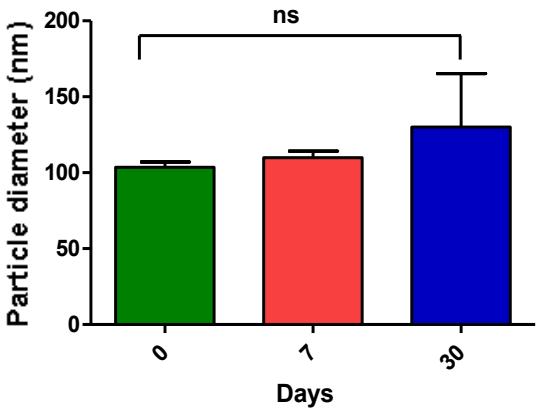


Fig. 3.1 (A-C) Effect of the amount of DHA-dFdC on the stability of the resultant DHA-dFdC-SLNs. Shown are particle size (A), polydispersity index (B), and zeta potential (C) of the resultant DHA-dFdC-SLNs after 6 days of storage at 4°C. Data shown are mean ± SD (n = 3). (D) A representative particle size distribution curve of DHA-dFdC-SLNs prepared with 5.2 mg of DHA-dFdC. (E) A representative TEM image of DHA-dFdC-SLNs prepared with 5.2 mg of DHA-dFdC (bar = 200 nm). (F) A presentative gel permeation chromatograph of DHA-dFdC-SLNs prepared with 5.2 mg of DHA-dFdC. DHA-dFdC-SLNs were applied to a Sepharose 4B column, and the elution fraction was 0.5 mL. D and F, experiments were repeated at least three times with similar results.

3.4.2 Chemical stability of DHA-dFdC in DHA-dFdC-SLNs after lyophilization

To select a proper lyoprotectant, we screened various sugars including sucrose, mannitol, and trehalose with concentrations ranging from 2.5% (w/v) to 5% (w/v) and found that sucrose at concentrations between 2.5% to 3% can effectively prevent particle size change after the DHA-dFdC-SLNs were subjected to lyophilization and reconstitution (data not shown). Sucrose at 3% (w/v) was thus used as the lyoprotectant for further studies. As shown in Fig. 3.2A, the particle size of the DHA-dFdC-SLNs did not significantly change after 30 days of storage as a lyophilized powder at room temperature. Importantly, the content of DHA-dFdC in the lyophilized DHA-dFdC-SLNs powder remained unchanged during the 30 days of storage (Fig. 3.2B). As a comparison, just 19.1% ± 7.0 of DHA-dFdC in the DHA-dFdC-vitamin E solid mixture was left after 14 days of storage in the same condition ($p < 0.0001$) (Fig. 3.2C). Previously, we reported that DHA-dFdC in a Tween 80-ethanol-water solution was unstable when storage at room temperature, with a half-life of ~14 h, while the addition of vitamin E (0.01%, w/v) in the solution helps to increase the stability of DHA-dFdC in the solution (5). The improved chemical stability of DHA-dFdC in the DHA-dFdC-SLNs dry powder may be attributed to the following three reasons. First, the SLNs may have protected DHA-dFdC incorporated in them from chemical degradation (9). For example, it was reported that β-carotene loaded in SLNs have improved stability because the β-carotene was protected against oxidation (9, 34). Second, the incorporation of TPGS in the formulation likely provided antioxidant properties since TPGS contains α-tocopherol or vitamin E, and TPGS was reported to have more antioxidant activity than free α-tocopherol (20, 21). Third, the SLNs were lyophilized into a dry powder (35-37).

(A)



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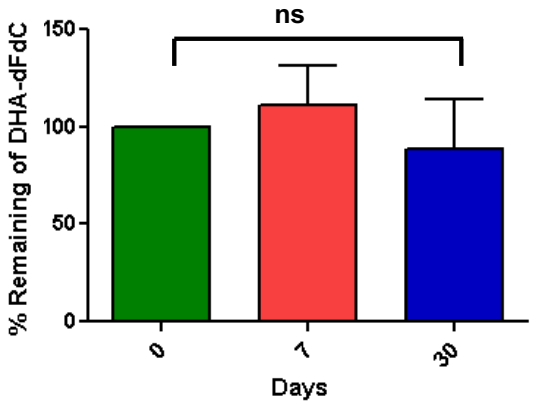

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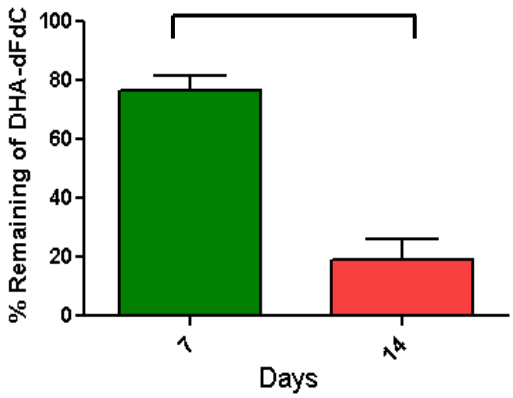


Fig. 3.2 Stability of DHA-dFdC and DHA-dFdC-SLNs as a lyophilized powder. (A) Particle size of DHA-dFdC-SLNs. (B) The concentration of DHA-dFdC remaining in the DHA-dFdC-SLNs. The measures in A and B were performed on 0, 7 and 30 days after the DHA-dFdC-SLNs were lyophilized and stored at room temperature. (C) Chemical stability of DHA-dFdC in a dry waxy solid that contains 5.047% (w/w) of vitamin E when stored at room temperature for 14 days. *** $p < 0.001$. Data are mean ± S.D. (n = 3).

3.4.3 *In vitro* characterization of DHA-dFdC-SLNs

The particles size of DHA-dFdC-SLNs after 18 h of incubation in a simulated biological medium (i.e. 10% FBS in PBS) at 37° C did not increase, suggesting that after intravenous administration, DHA-dFdC-SLNs will not likely aggregate. Shown in Fig. 3.3 is the release profile of DHA-dFdC from the DHA-dFdC-SLNs. Only 8.6% ± 1.9 of DHA-dFdC was released from the SLNs within 8 h.

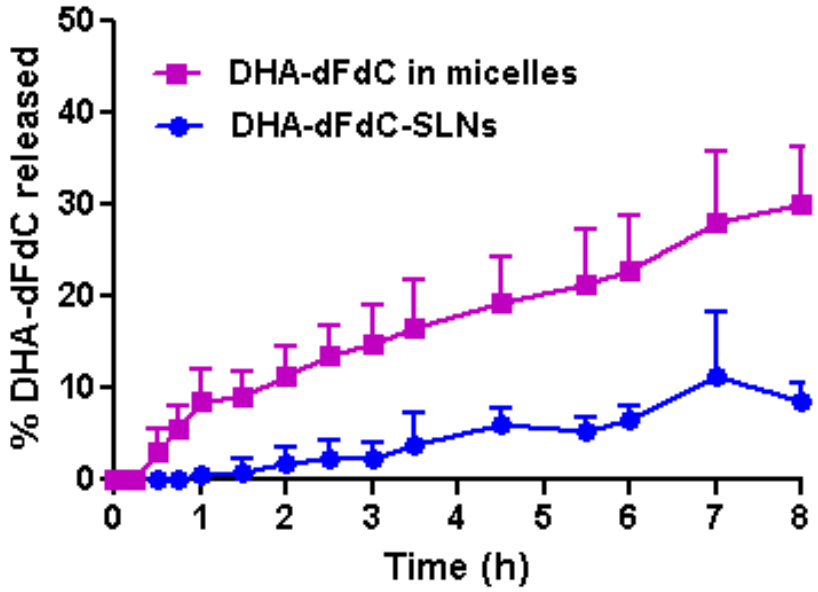
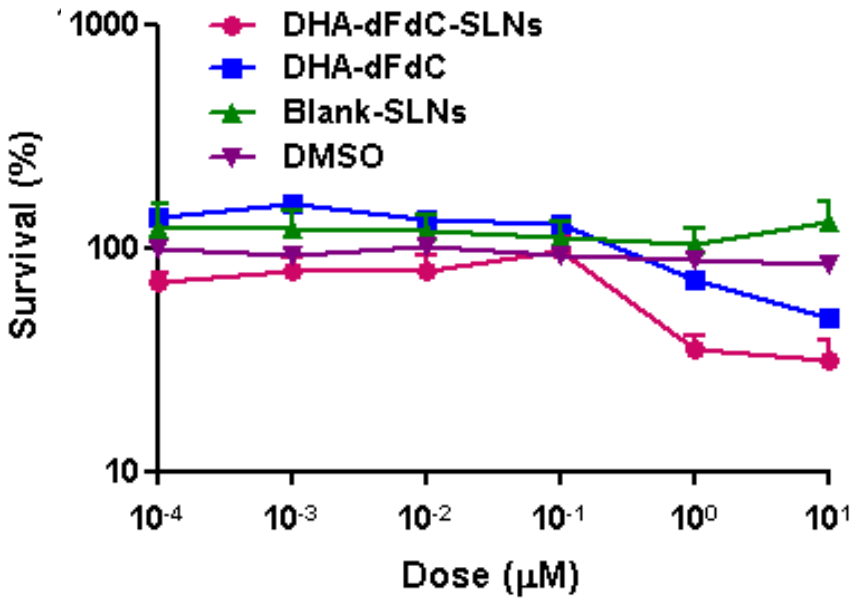


Fig. 3.3 *In vitro* release profile of DHA-dFdC from DHA-dFdC-SLNs. The diffusion of DHA-dFdC (in Tween 20 micelles) across the dialysis membrane was measured as well. Data are mean ± SD (n = 3).

3.4.4 Evaluation of the cytotoxicity of the DHA-dFdC-SLNs against tumor cells in culture

The cytotoxicity of the DHA-dFdC-SLNs was evaluated by determining the survival of tumor cells after incubation with the SLNs using the MTT assay. As shown in Fig. 3.4A-B, DHA-dFdC-SLNs were more cytotoxic than DHA-dFdC in M-Wnt (i.e. IC_{50} values of 0.92 μM versus 2.15 μM, $p < 0.05$, 24 h of incubation) and B16F10 cells (i.e. IC_{50} values of 0.085 μM versus 1.81 μM, $p < 0.0001$, 48 h of incubation). In TC-1 cells, the cytotoxicity of DHA-dFdC-SLNs was not significantly different from that of DHA-dFdC (Fig. 3.4C). DHA-dFdC-free SLNs and dimethyl sulfoxide (i.e. vehicle used to dissolve DHA-dFdC) did not show significant cytotoxicity in the concentrations tested in all three cell lines (Fig. 3,4).

(A)



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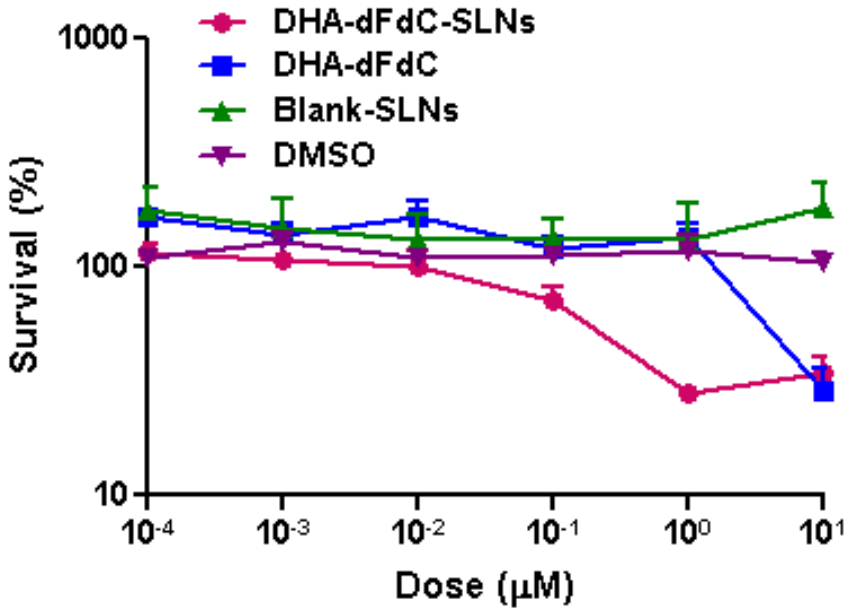
(B)



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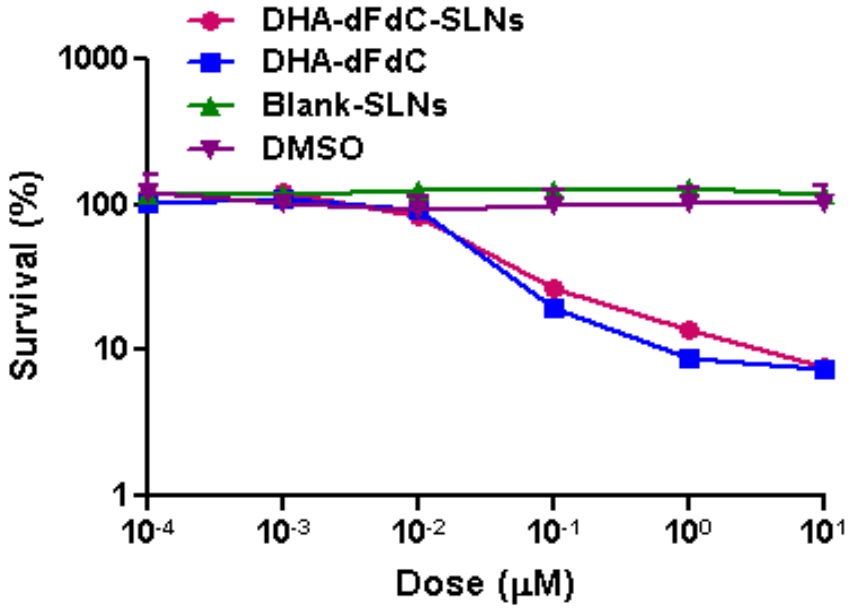


Fig. 3.4 Cytotoxicity of DHA-dFdC-SLNs in M-Wnt cells (A), B16-F10 cells (B), and TC-1 cells (C). In A, the cells were incubated with the nanoparticles for 24 h, 48 h for B and C. As controls, cells were also incubated with DHA-dFdC-free SLNs, DHA-dFdC dissolved in DMSO or the equivalent concentration of DMSO, or cell culture media alone. Data shown are mean ± SD ($n > 3$).

Data from previous studies support that TPGS improve the activity of nanoparticles by enhancing cell uptake and/or increasing cytotoxicity (16, 19, 22). For example, high cellular uptake and cytotoxicity of docetaxel were reported when docetaxel-loaded liposomes were coated with TPGS, as compared to PEGylated liposomes without TPGS (19). Other TPGS-emulsified nanoparticles or TPGS-based nanoparticles were also showed to have high cellular uptake and cytotoxicity in cell lines such as Caco-2, HT-29, MCF-7, C6 gliomas cells (16). In addition, some studies showed a synergic effect between TPGS and docetaxel, doxorubicin, and paclitaxel (23, 24, 26). For DHA-dFdC, we have reported that it has potent cytotoxicity against many tumor cells, including the TC-1 cell, which may explain that incorporating it into SLNs may not necessarily further improve its cytotoxicity against all cell lines in culture (5).

3.4.5 Plasma pharmacokinetic of DHA-dFdC in DHA-dFdC-SLNs

Data in Fig. 3.5 showed the plasma DHA-dFdC levels in mouse plasma samples at different time points after intravenous injection of DHA-dFdC-SLNs. The elimination of DHA-dFdC in mouse plasma follows a bi-exponential model. Table 3.2 includes selected PK parameters of DHA-dFdC. The $AUC_{0-\infty}$ values for DHA-dFdC was 677.3 μg/ml*h, and the plasma half-life of DHA-dFdC in the elimination phase was ~44 h. For a comparison, previously, we reported that when DHA-dFdC was given in a Tween 80-ethanol-water solution to mice, its plasma half-life was only ~58 min (5).

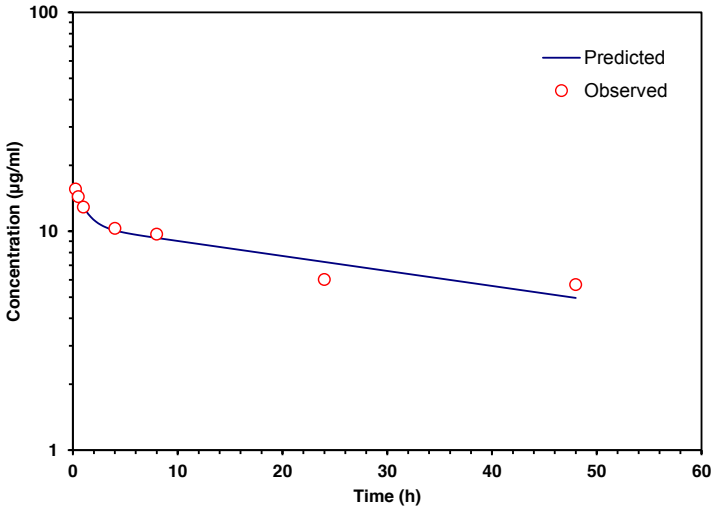


Fig. 3.5 Plasma DHA-dFdC concentration (µg/mL) at different time points (h) after DHA-dFdC-SLNs in suspension were intravenously injected into in C57BL/6 mice. The dose of DHA-dFdC was 2 mg per mouse. Data were fitted using PKSolver, assuming a two-compartment model.

Table 3.2 Plasma PK parameters of DHA-dFdC-SLNs when given intravenously to mice

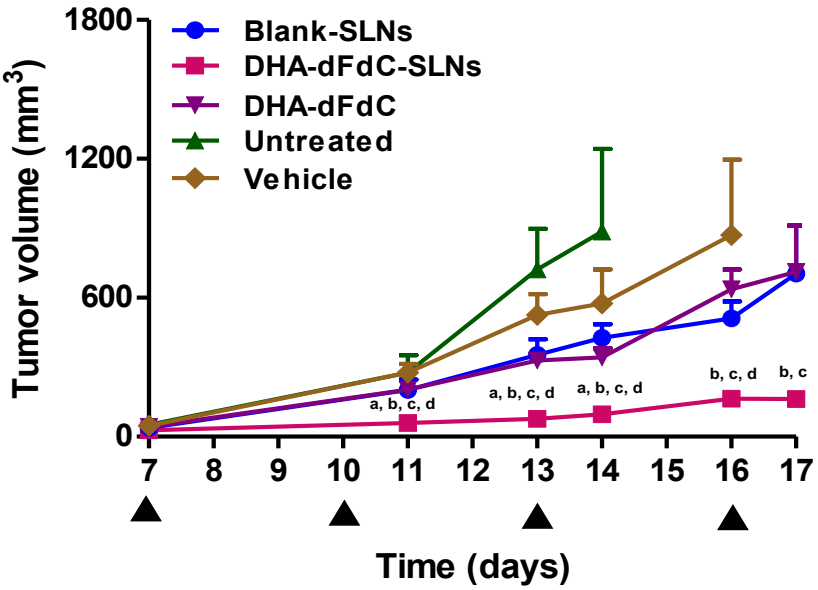
Parameter	Unit	Observed
k10	1/h	0.02
k1/2	1/h	0.32
k21	1/h	0.58
t1/2α	h	0.76
t1/2β	h	43.95
C0	µg/ml	16.85
V	ml	0.12
CL	ml/h	0.03
V2	ml	0.07
CL2	ml/h	0.04
$AUC_{0\text{-}24h}$	(µg/ml)*h	362.82
$AUC_{0\text{-}inf}$	(µg/ml)*h	677.30
AUMC	(µg/ml)*h^2	42519.06
MRT	h	62.78

3.4.6 Evaluation of the antitumor activity of DHA-dFdC-SLNs in mouse model

The antitumor activity of DHA-dFdC-SLNs was evaluated in mice with pre-established B16-F10 tumors. As shown in Fig. 3.6A, tumors grew aggressively when mice were left untreated or treated with the Tween 80-ethanol-in-water vehicle only. DHA-dFdC in solution and Blank-SLNs (free-DHA-dFdC-SLNs) at the dosing regimen tested delayed the tumor growth by 4 days, but there were not any significant difference between the sizes of the tumors in mice treated with DHA-dFdC in solution or Blank-SLNs and the sizes of tumors in mice left untreated in all the days compared (Fig. 3.6A). DHA-dFdC-SLNs were most effective in inhibiting the tumor growth; the nanoparticle formulation delayed the tumor growth by close to 8 days, and the sizes of the tumors in mice that were treated with the DHA-dFdC-SLNs were significantly smaller than those in mice that were left untreated or treated with the DHA-dFdC in solution (Fig. 3.6A). There was not any significant difference in the body weights of mice among the groups during the treatments (Fig. 3.6B), indicating DHA-dFdC-SLNs at the dosing regimen tested were well tolerated. Shown in Fig. 3.7 are representative H&E images of B16-F10 tumors from mice in different groups. Tumors in mice that were left untreated (A) or treated with vehicle (B) or DHA-dFdC-free SLNs (C) were in a late tumor stage with large blood vessels with large lumen. In addition, tumors in these groups showed large necrotic area, increase in the desmoplasia, and vascular collapse (Fig. 3.7A-C). In solid tumors such as melanoma, high interstitial fluid constitutes a significant barrier to chemotherapy as it induces the compression of blood vessels, diverting the blood from the center of tumors to the periphery, which reduces the transcapillary transport of chemotherapeutics (38). Tumor treated with DHA-dFdC-SLNs showed a higher number of blood vessels with small lumen (Fig. 3.7G). In addition, an increasing level of connective tissue can be observed around the tumoral zone in tumors in mice treated with DHA-dFdC-SLNs (Fig. 3.7F). In fact, this connective tissue may have tumor encapsulation effect, providing a protective barrier to tumor local and vascular invasion as suggested (39). For example, it was reported that patients with liver metastasis have a better prognostic when metastasis encapsulation occurs by the formation of a fibrotic

capsule (40-42). Indeed, it was suggested that the formation of capsules protects the liver parenchyma from cancer invasion (42). This evidence supports the use of DHA-dFdC-SLNs to treat melanoma, inducing the tumor encapsulation for further removal by surgery and avoiding metastasis due to the protective effect of this capsule. On the other hand, tumors in mice treated with DHA-dFdC showed vascular collapse, high desmoplasia, and necrotic areas (Fig. 3.7D-E). Tumors in mice treated with DHA-dFdC-SLNs shown a more cells in apoptosis, but less cells in necrosis, as compared to tumors in mice treated with DHA-dFdC in solution or left untreated. In this study, DHA-dFdC-free SLNs (i.e. Blank-SLNs) showed a tendency to delay tumor growth as compared to the untreated group (Fig. 3.6A). In fact, *in vivo* and *in vitro* studies reported that TPGS had anticancer activity as a single agent, being able to inhibit the growth of human prostate and lung carcinoma cells (25, 43). Furthermore, it was reported that TPGS selectively induces apoptosis in T cell acute lymphocytic leukemia (ALL) or Jurkat clone E6-1 cells through the induction of oxidative stress pathway (44). In addition, TPGS was reported to selectively induce cell cycle arrest and apoptosis in breast cancer cell lines such as MCF7 and MDA-MB-231, but not in "normal" immortalized cells such as MCF-10A and MCF-12F (45). Finally, a synergistic effect between $TPGS_{2k}$ and docetaxel was reported in MCF-7 cell lines, wherein the incubation of MCF-7 cells with $TPGS_{2k}$ micelles without docetaxel induced cytotoxicity (24). One reason that could explain the lack of cytotoxicity by our DHA-dFdC-free SLNs in culture cells is the low concentration of TPGS used in the formulation (≈ 1.5 mM). Indeed, higher concentrations TPGS were used in culture to induce cell cytotoxicity (e.g. > 10 mM), and in animal studies to suppress tumor growth (≈ 40 mM) (25, 43-45).

(A)



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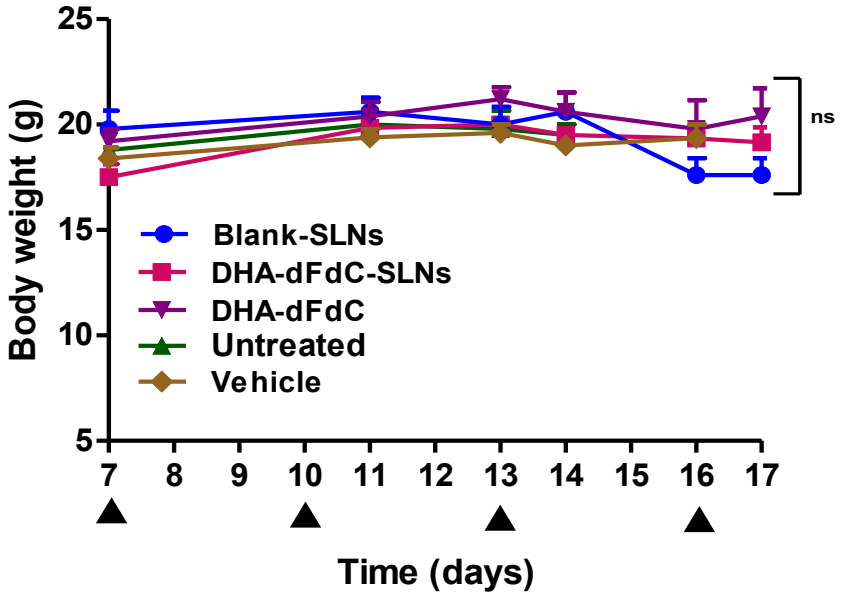

Fig. 3.6 Antitumor activity of DHA-dFdC-SLNs against B16-F10 tumor in a mouse model. (A) Tumor growth curve. (B) Mouse body weight change curves. C57BL76 mice were s.c. injected with B16-F10 tumor on day 0. On day 7, mice were randomized into 5 groups (n = 5-6) and i.v. injected with DHA-dFdC-SLNs, DHA-dFdC in vehicle, Blank-SLNs (DHA-dFdC-free SLNs) on days 7, 10, 13, and 16. The dose of DHA-dFdC was 50 mg/kg. As controls, one group of mice were left untreated. Data shown are mean ± SEM. $p < 0.05$; a DHA-dFdC-SLNs vs untreated; b DHA-dFdC-SLNs vs DHA-dFdC; c DHA-dFdC-SLNs vs Blank-SLNs; d DHA-dFdC-SLNs vs vehicle

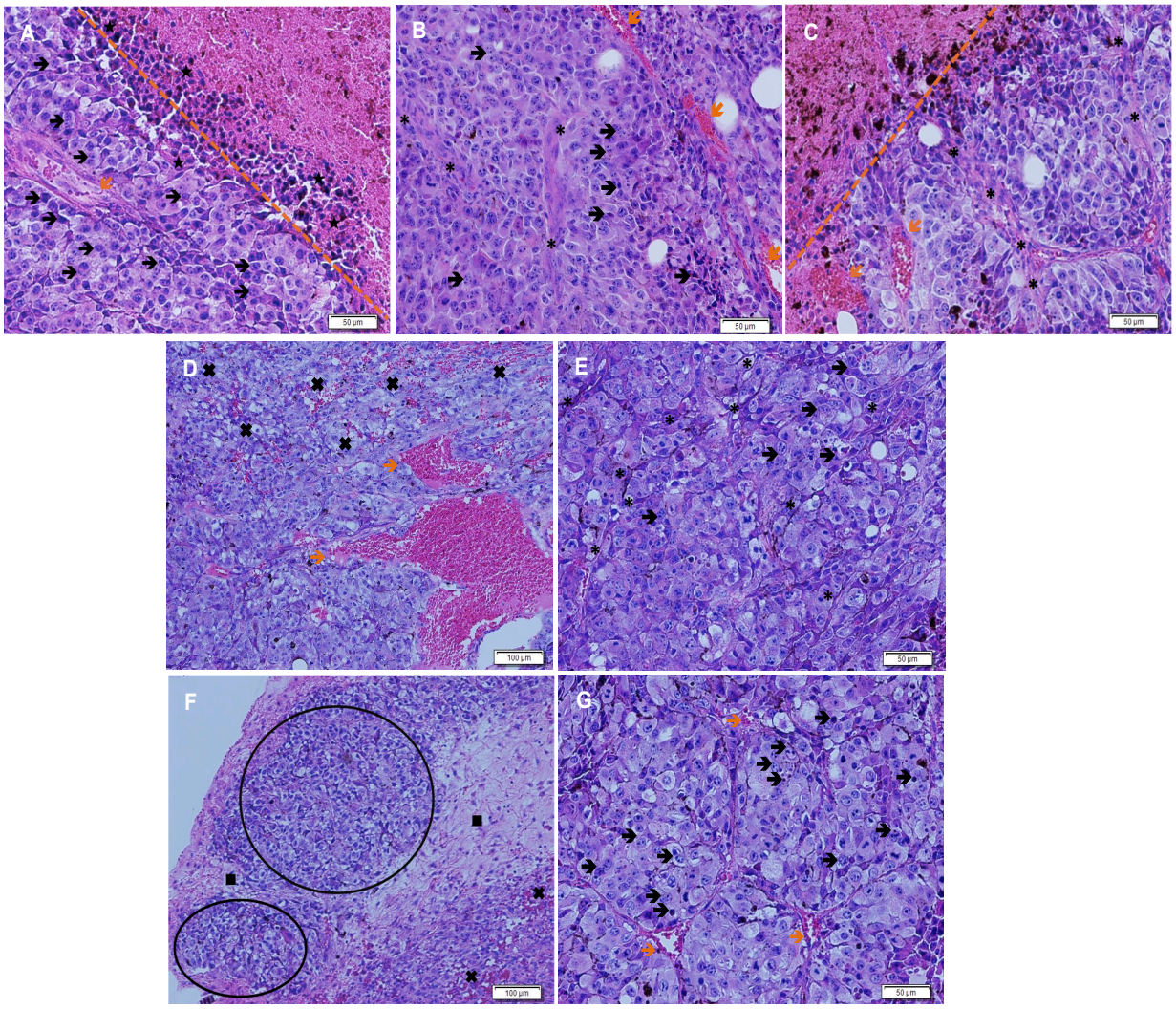

Fig. 3.7 (A-G) Representative H&E images of B16-F10 tumors in C57BL76 mice i.v. injected with DHA-dFdC-SLNs, DHA-dFdC-free SLNs, DHA-dFdC in vehicle, vehicle alone or left untreated. Mice were euthanized on day 17 to collect tumor tissues. Tumor tissues of untreated (A), vehicle (B), and Blank-SLNs (C) groups are represented at a magnification 200X; while DHA-dFdC (D-E) and DHA-dFdC-SLNs (F-G) groups are represented by two different magnifications (100X (left), 200X (right)). The scale bars in the 100 X images represent 100 μm, and that in the 200 X images represent 50 μm. Black circles represent tumor area, orange dashed lines represent necrotic area, black arrows represent apoptotic cells, asterisk represent desmoplasia, orange arrows represent blood vessel, times signs represent infiltration areas, black squares represent connective tissue areas, and stars represent necrotic cells.

In summary, we report a promising SLN formulation of our DHA-dFdC. All materials used in the formulation are biocompatible. Indeed, lecithin, GMS, and Tween 20 are GRAS materials for parenteral administration (46). TPGS has been approved by the FDA as a safe pharmaceutical adjuvant that allows its use parenteral pharmaceutical formulations (46). Moreover, the method of preparing the SLN formulation is straight forward, inexpensive and potentially scalable for industrial manufacturing. In addition, the small size of the DHA-dFdC-SLNs (102.2 ± 7.3 nm) allows their sterilization by filtration (0.2 μm). Finally, toxic organic solvents were not used when preparing the SLNs during the emulsion preparation, avoiding the evaporation process and residual solvent in the formulation. As to the mechanism underlying the improved antitumor activity of the DHA-dFdC-SLNs in the animal model, the enhance permeability and retention effect (EPR) was likely responsible (47).

3.5 CONCLUSION

We report a new solid lipid nanoparticle formulation of DHA-dFdC that significantly increases the water solubility and chemical stability of DHA-dFdC. The DHA-dFdC-SLNs also favorably modify the plasma pharmacokinetics of the DHA-dFdC and enhance its antitumor activity.

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Chapter 4: A Solid Lipid Nanoparticle Formulation Improves the Oral Bioavailability of 4-(*N*)-Docosahexaenoyl 2′, 2′-Difluorodeoxycytidine

4.1 ABSTRACT

Previously, we synthesized 4-(*N*)-docosahexaenoyl 2′, 2′-difluorodeoxycytidine (DHA-dFdC), a novel lipophilic compound with a potent, broad spectrum antitumor activity and developed a solid lipid nanoparticle (SLN) formulation of the DHA-dFdC (i.e. DHA-dFdC-SLNs) to improve the solubility and chemical stability of DHA-dFdC. The SLNs were prepared from lecithin/glycerol monosterate-in-water emulsions emulsified with D-α-tocopherol polyethylene glycol 1000 succinate and Tween 20. Herein, we studied the feasibility of administering the DHA-dFdC by the oral route using the SLN formulation. In simulated gastrointestinal fluids, the DHA-dFdC-SLNs did not aggregate. Overall, the release of the DHA-dFdC from the SLNs in simulated gastrointestinal fluids was slow, but it was slightly faster in simulated intestinal fluid than in simulated gastric fluid. In mice orally gavage with DHA-dFdC-SLNs, plasma DHA-dFdC concentration followed a bi-exponential model, with a T_{max} of ~ 1.7 h and a C_{max} of 17.01 μg/mL. The absolute oral bioavailability of DHA-dFdC in the DHA-dFdC-SLNs was ~ 68% (based on the $AUC_{0\text{-}24h}$ values). Finally, in mice with pre-establish B16-F10 murine melanoma, oral DHA-dFdC-SLNs increased the mouse survival significantly. It is concluded that the DHA-dFdC-SLNs can be used to safely administer DHA-dFdC by the oral route.

4.2 INTRODUCTION

Oral route is preferred for drug administration due to advantages such as painlessness, easiness for self-administration, flexibility in dosage regimen, convenience, and high patient compliance (1, 2). Furthermore, oral product manufacturing does not require sterile conditions that are necessary for products intended for parenteral administration (1). In cancer chemotherapy, there are studies reported that cancer patients prefer oral administration to intravenous infusion, especially when chemotherapy is just a palliative treatment (2-4). However, oral administration of cancer chemotherapeutic agents is rather challenging, in part because the gastrointestinal (GI) tract presents various physiological, enzymatic and chemical barriers, hindering an efficient oral absorption (2, 5). In addition, factors such as low solubility, poor intestinal permeability, and high level of P-glycoprotein (P-gp) in the GI tract wall also limit the oral bioavailability of many cancer chemotherapeutic agents (e.g., paclitaxel, docetaxel, doxorrubicin, tamoxifen, etc.) (2). Nanocarriers (e.g. SLNs, liposomes, nanoemulsions, micelles, and polymeric nanoparticles) have gained attention to improve the oral delivery of anticancer drugs due to their ability to increase the apparent solubility of drugs, reduce the degradation of drugs within the GI tract, and improve drug absorption (1, 2, 5).

Previously, we reported a new SLN formulation of DHA-dFdC, DHA-dFdC-SLNs, to improve the apparent solubility and stability of DHA-dFdC, a novel compound synthesized in our laboratory by conjugating docosahexaenoic acid at the 4-NH_2 position of dFdC (2′, 2′-difluorodeoxycytidine). DHA-dFdC shows a potent and broad-spectrum antitumor activity *in vivo* and *in vitro* (6). DHA-dFdC-SLNs were prepared by incorporating DHA-dFdC into SLNs prepared from lecithin/glycerol monostreate (GMS)-in-water emulsions emulsified with Tween 20 and D-α-tocopherol polyethylene glycol 1000 succinate (vitamin E TPGS or TPGS). The SLNs are 102 ± 7 nm, with a zeta potential of -55 ± 3 mV. DHA-dFdC-SLNs increase the apparent water

solubility and chemical stability of DHA-dFdC. In the present work, we show that the DHA-dFdC-SLNs also enable the oral administration of DHA-dFdC.

4.3 MATERIALS AND METHODS

4.3.1 Materials and cell lines

Mannitol, Tween 20, GMS, TPGS, sodium chloride (NaCl), hydrochloric acid (HCl, 37 %), monobasic potassium phosphate (KH_2PO_4), sodium hydroxide (NaOH), and Tween 80 were from Sigma-Aldrich (St. Louis, MO). Gemcitabine HCl was from Biotang, Inc. (Lexington, MA). Soy lecithin was from Alfa Aesar (Ward Hill, MA). Ethyl acetate (EtOAc), tetrahydrofuran (HPLC-grade), isopropanol, and methanol (HPLC-grade) were from Thermo Fisher Scientific (Waltham, MA). Float-A-Lyzer®G2 dialysis device (MWC 50 kD) was from Spectrum Inc. (New Brunswick, NJ).

Murine melanoma (B16-F10) cancer cell lines were from the American Type Culture Collection (Manassas, VA). B16-F10 cells were grown in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/mL of penicillin, and 100 μg/mL of streptomycin, all from Invitrogen (Carlsbad, CA).

4.3.2 Synthesis of 4-(*N*)-docosahexaenoyl 2′,2′-difluorodeoxycytidine (DHA-dFdC)

DHA-dFdC was synthesized following our previously reported conjugation scheme (6). The purity of the resultant DHA-dFdC was confirmed by NMR and Mass Spectrum analyses.

4.3.3 Preparation and characterization of 4-(*N*)-docosahexaenoyl 2′, 2′-difluorodeoxycytidine nanoparticles (DHA-dFdC-SLNs)

Nanoparticles were prepared as previously described (7). Briefly, 3.5 mg of soy lecithin, 0.5 mg of GMS, 0.875 mg of TPGS, were weighed into a 7 ml glass vial. Eight hundred microliter of de-ionized and filtered (0.22 μm) hot water (at 80°C) was added into the lecithin/GMS/TPGS mixture, which was then vortexed and sonicated for 3 min until homogenous slurry was formed. The mixture was maintained on an 80°C hot plate for 5 min. A solution of Tween 20 (55 mg in 1 ml of water) was prepared, and 200 μL of the solution was added drop wise into the mixture to reach a final concentration of 1% (v/v). The resultant emulsions were allowed to cool to room temperature while stirring to form nanoparticles.

To incorporate DHA-dFdC into the nanoparticles to form DHA-dFdC-SLNs, 5.2 mg of DHA-dFdC was added into lecithin/GMS/TPGS mixture before the addition of water. The remaining steps were identical to the preparation of DHA-dFdC-free nanoparticles. The size and zeta potential of the nanoparticles were measured using a Malvern Zetasizer Nano ZS (Westborough, MA).

DHA-dFdC was extracted from the nanoparticles to determine its concentration. Briefly, 100 μL of DHA-dFdC-SLNs were mixed with 100 μL of isopropanol, vortexed for 30 s, and maintained at room temperature. Five minutes later, 600 μL of ethyl acetate was added. The mixture was vortexed per 30 s and centrifuged at 11,000 rcf for 20 min. The supernatant was collected into a glass vial. After the solvent was evaporated under nitrogen, the sample was re-dissolved in 100 μL of THF, and the concentration of DHA-dFdC was measured by HPLC (6).

4.3.4 Stability of DHA-dFdC-SLNs in stimulated gastrointestinal fluids

The stability of DHA-dFdC-SLNs in simulated gastric fluid (SGF, pH 1.2) and simulated intestine fluid (SIF, pH 6.8) without enzymes was evaluated. SGF and SIF were prepared according USP XXVI. The SGF was prepared by dissolving 2 g of NaCl into 7 mL of HCl, and completed the volume to 1000 mL with deionized water (8). The SIF was prepared by adding 6.8

g of KH_2PO_4 and 896 mg NaOH into 1000 mL of deionized water (8). DHA-dFdC-SLNs were incubated in SGF or SIF media at 37°C under agitation (100 rpm). At different time points (i.e. 0, 1, 2, 4, and 6 h), samples were taken and diluted into water to measure particle size using Malvern Zetasizer Nano ZS. As a control, DHA-dFdC-SLNs were incubated in phosphate-buffered saline (PBS, 10 mM, pH 7.4). The experiments were repeated three times.

4.3.5 Transmission electron micrographs (TEM)

The size and morphology of the DHA-dFdC-SLNs before and after incubation in SGF and SIF were examined using a transmission electron microscope available in the Institute for Cellular and Molecular Biology Microscope and Imaging Facility at The University of Texas at Austin. The carbon film-coated copper grid was glow discharged for 2 min. A sample of 10 μL of DHA-dFdC-SLNs suspended in water was deposited on the grid and left to stand for 1 min. The sample excess was removed with a filter paper. One drop of 1% uranyl acetate was added on the grid for 30 s. The sample was then observed under the TEM after removing the excess uranyl acetate fluid with filter paper (9).

4.3.6 *In vitro* release in simulated gastrointestinal fluids

To test the release behavior of DHA-dFdC from DHA-dFdC-SLNs in SGF and SIF, DHA-dFdC-SLNs in SGF or SIF were placed into a 1 mL of cellulose ester dialysis tube (151 μg/mL of DHA-dFdC), which was then placed in a plastic conical tube containing 13 mL of dissolution media (i.e. SGF or SIF with 2.5% of Tween 20) to create a sink condition. The plastic tube was placed in a thermostatic shaker at 37°C at 100 rpm (Max Q 200, Thermo Fisher Scientific). At predetermined time points, 200 μL of the release medium was withdrawn and subsequently replaced with an equal volume of fresh medium. The concentration of DHA-dFdC in the medium was determined using HPLC. As a control, 151 μg of DHA-dFdC was dissolved in 2.5% of Tween

20 to confirm that the diffusion of DHA-dFdC across the dialysis tube membrane was not rate-limiting.

4.3.7 Pharmacokinetic studies

The Institutional Animal Care and Use Committee at The University of Texas at Austin approved the animal protocol. Female C57BL/6 mice (6-8 weeks, Charles River Laboratories, Wilmington, MA) were fasted for 3 h. Water was allowed ad libitum. Mice were orally gavaged with DHA-dFdC dissolved in a vehicle solution (i.e. Tween 80 (10%, w/v), ethanol (5.2% v/v), and mannitol (5%, w/v) in sterile water) (6, 10) or the DHA-dFdC-SLNs suspended in a sterile mannitol solution (5%, w/v), or intravenously injected with the DHA-dFdC-SLNs suspended in a sterile mannitol solution (5%, w/v). The dose of DHA-dFdC was 2 mg per mouse. Mice (n = 3) were euthanized at various time points (i.e. 0.25, 0.5, 1, 2, 5, 8, 12, and 24 h). Blood was collected into heparin-coated tubes, which were then centrifuged at 13,000 rcf for 20 min to isolate plasma. The plasma (200 μL) was mixed with 200 μL of isopropanol and 200 μL of cold PBS, vortexed and then incubated at 4°C for 5 min. Following incubation, 1000 μl of ethyl acetate was added. The mixture was vortexed for 5 min, followed by centrifugation at 18,000 rcf for 5 min. The supernatant was collected and dried under nitrogen. Finally, the residue was re-dissolved in 100 μl of THF, which was then analyzed using HPLC (6). As internal control 4-(*N*)-stearoyl 2′,2′-difluorodeoxycytidine (GemC18) was added in the samples before extraction (11). Data were analyzed using PK Solver®, assuming a two-compartmental model (8).

4.3.8 Antitumor activity of orally administered DHA-dFdC-SLNs in a tumor-bearing mouse model

Female C57BL/6 mice (18-20 g) were subcutaneously (s.c) injected with B16-F10 (5×10^5 cells/mouse) in the right flank on day 0. Seven days later, mice were randomized into 4 groups (n = 7-8) and orally gavaged with DHA-dFdC (250 μg/mouse) dissolved in vehicle (6, 10), DHA-

dFdC-SLNs (250 μg/mouse of DHA) dispersed in mannitol 5%, or DHA-dFdC-free SLNs dispersed in mannitol 5%. As a control, one group of mice were left untreated. Treatment was repeated every day until day 11. Mice were allowed to rest for two days, and treatment was resumed on day 13 and continued until day 20. Mice were monitored daily until the endpoint (i.e. death, tumor size reaching 15 mm, tumor ulceration, body weight loss of more than 20%, or other signs of severe distress and discomfort).

4.3.9 Statistical analysis

Statistical analyses were completed by one-way ANOVA followed by a Bonferroni post hoc test. Mouse survival curves were compared using the Mantel-Cox log-rank method. A p value of ≤ 0.05 (two-tail) was considered significant. Most of the analyses were performed with GraphPad Prism (GraphPad Software, Inc., La Jolla, CA). Pharmacokinetic parameters were obtained using PK Solver® (12).

4.4 RESULTS AND DISCUSION

The use of solid-lipid nanoparticles for oral drug administration provides several advantages, such as improving the stability, enhancing the bioavailability of the drug and decreasing its toxicity (5, 13-16). Previously, we prepared DHA-dFdC-SLNs by incorporating DHA-dFdC into solid lipid nanoparticles prepared with soy lecithin, GMS, TPGS, and Tween 20 to overcome its poor water solubility and chemical instability of DHA-dFdC (paper submitted) (6). The main characteristics of the DHA-dFdC-SLNs are summarized in Table 4.1. The size of the nanoparticles is 100.5 ± 7.72 nm. It is known that the particles size significantly affect the gastrointestinal absorption, and nanoparticles with a particle size lower than 300 nm are good candidate for oral administration (2, 17). Indeed, an evaluation of the cellular uptake of polymeric nanoparticles (i.e. Vitamin E TPGS-coated PLGA nanoparticles or PVA-coated PLGA nanoparticles) by Caco-2 cells in culture showed that the most desirable particles size is in the range of 100-200 nm (17). It was also reported that the surface properties of the nanoparticles are also important to improve the cellular uptake of the nanoparticles; surface modification of the nanoparticles with vitamin E TPGS improved considerably the cellular uptake (17). The zeta potential of our DHA-dFdC-SLNs is -43.5 ± 2.2 mV, indicating their stability in an aqueous suspension (17, 18).

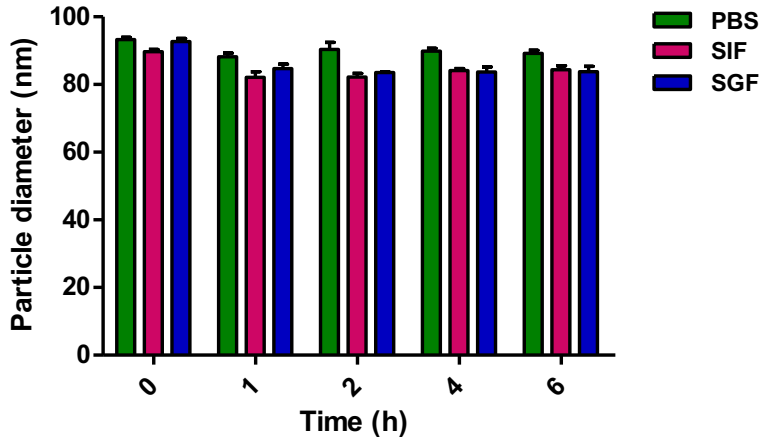
Table 4.1 Characterization of DHA-dFdC-SLNs. Data shown are mean ± S.D. (n = 3)

DHA-dFdC (mg)	5.2
Particle diameter (nm)	100.5 ± 7.72
Polydispersity index	0.214 ± 0.03
Zeta potential (mV)	-43.5 ± 2.2
Entrapment efficiency %	97% ± 21.4

4.4.1 Stability of DHA-dFdC-SLNs in stimulated gastrointestinal fluids

Figure 4.1 shows the *in vitro* stability data of DHA-dFdC-SLNs in simulated gastrointestinal (GI) fluid (e.g. SGF or SIF). As a control, the stability of the DHA-dFdC-SLNs in PBS (10 mM, pH 7.4) was also included. The particles size of the DHA-dFdC-SLNs as measured by DLS did not increased during a 6 h of incubation in SGF or SIF. Indeed, particle size decreased slightly (i.e. ~5.4% in SIF and 6.1% in SGF, as compared to in PBS) (Fig. 4.1A). Shown in Fig. 4.1B-G are representative TEM images of the nanoparticles before and after 6 h of incubation in SGF or SIF. Overall, the shape of the nanoparticles did not change significantly after the incubation; however, after 6 h of incubation in SIF, the surface of the DHA-dFdC-SLNs appears rough (Fig. 4.1E, inset), which is not the case after the DHA-dFdC-SLNs were incubated in the SGF (Fig. 4.1G, inset). Studies examining the degradation of SLNs in GI fluids showed that their degradation induces a decrease in particle size due to the loss of surfactant coated on the surface of the nanoparticles, which ultimately led to an increase in the particle size of SLNs, because the nanoparticles aggregate in the absence of surfactant (18, 19). It was reported that non-ionic surfactants such as Tween 80, Tween 20, Tween 60, and PVA provide steric stabilization to particles in acid pH (20). Tween 20 was used as a surfactant in the DHA-dFdC-SLNs, which might explain the stability of them in the SGF. TPGS is a non-ionic surfactant as well, and the presence of TPGS in our DHA-dFdC-SLNs may have also contributed to the stability of the nanoparticles in simulated GI fluids. It is worth noting that the SGF and SIF we used in the stability studies did not contain enzymes. The nanoparticles will likely be less stable if enzymes were included in the fluids.

(A)



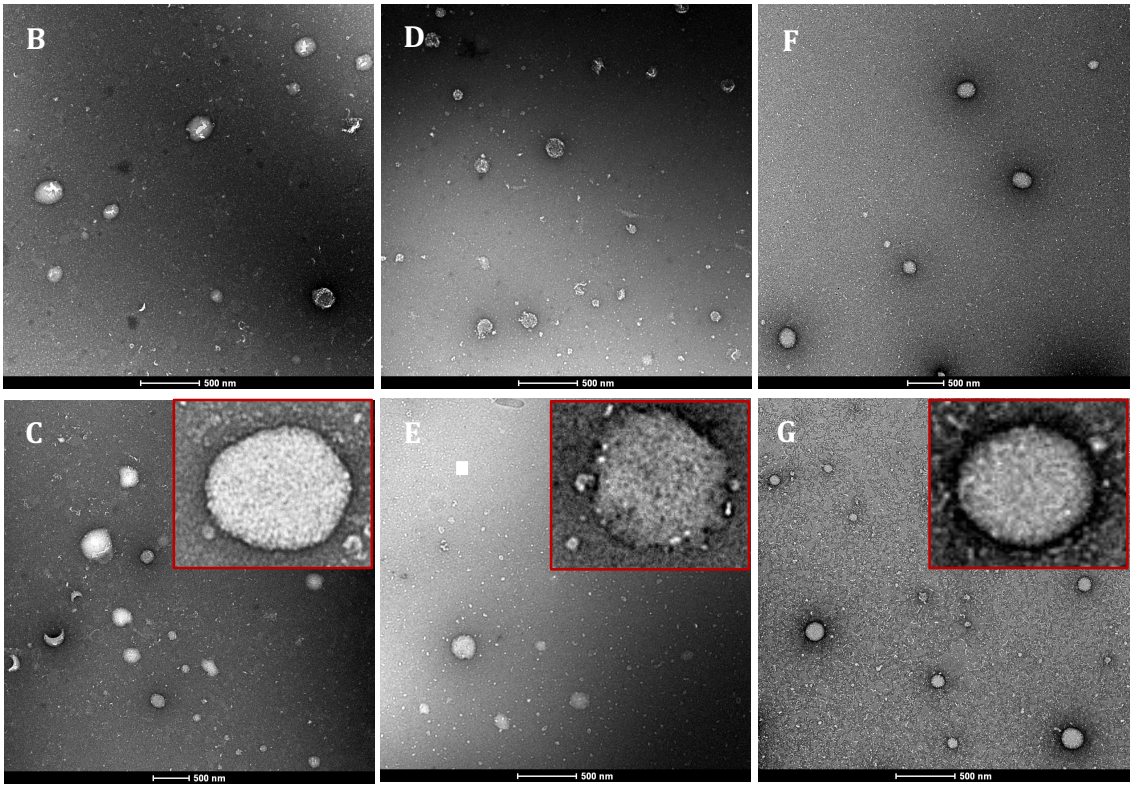


Fig. 4.1 Stability of DHA-dFdC-SLNs in simulated gastrointestinal fluids. A. Stability of DHA-dFdC-SLNs after 6 DHA-dFdC-SLNs were incubated with SGF (pH 1.2) or SIF (pH 6.8) at 37°C. Samples were collected at 0, 1, 2, 4 and 6 h to measure the particle size. As a control, DHA-dFdC-SLNs were also incubated with PBS. Data are expressed as mean ± SD (n = 3). B-G. Representative TEM images of DHA-dFdC-SLNs incubated with PBS (B-C at 0 and 6 h, respectively), SIF (D-E at 0 and 6 h, respectively), and SGF (F-G at 0 and 6 h, respectively). Bar = 500 nm

4.4.2 *In vitro* release in simulated gastrointestinal fluids

The *in vitro* release profiles of DHA-dFdC from the DHA-dFdC-SLNs in simulated GI fluids are shown in Fig. 4.2. After 6 h, the cumulative release of DHA-dFdC reached ~ 8.9% and ~ 3.2% in SIF and SIG, respectively. Similar to the stability study mentioned above, the release of DHA-dFdC from the DHA-dFdC-SLNs was monitored for 6 h only, because there is report that the GI transition time in mice is 6-8 h (21). As shown in Fig. 4.1E insert, the surface of the SLNs is not smooth after 6 h of incubation in SIF, indicting erosion of the particles, which may explain the faster release of DHA-dFdC from the SLNs in SIF.

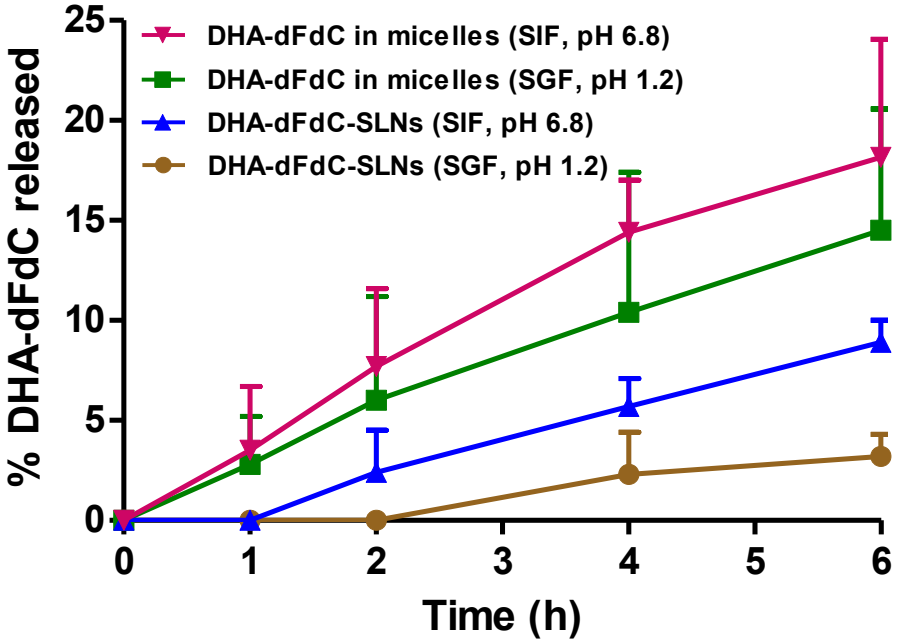


Fig. 4.2 *In vitro* release profiles of DHA-dFdC from DHA-dFdC-SLNs in simulated gastrointestinal fluids. As controls, the diffusion of DHA-dFdC (in DHA-dFdC-in Tween 20 micelles) across the dialysis membrane was also monitored. Data are expressed as mean ± SD (n = 3)

4.4.3 Oral bioavailability of DHA-dFdC in DHA-dFdC-SLNs

The plasma concentrations of DHA-dFdC at different time points after oral administration or intravenous injection of the DHA-dFdC-SLNs in suspension at 2 mg of DHA-dFdC per mouse are shown in Fig. 4.3. Selected pharmacokinetic parameters of DHA-dFdC are summarized in Table 4.2.

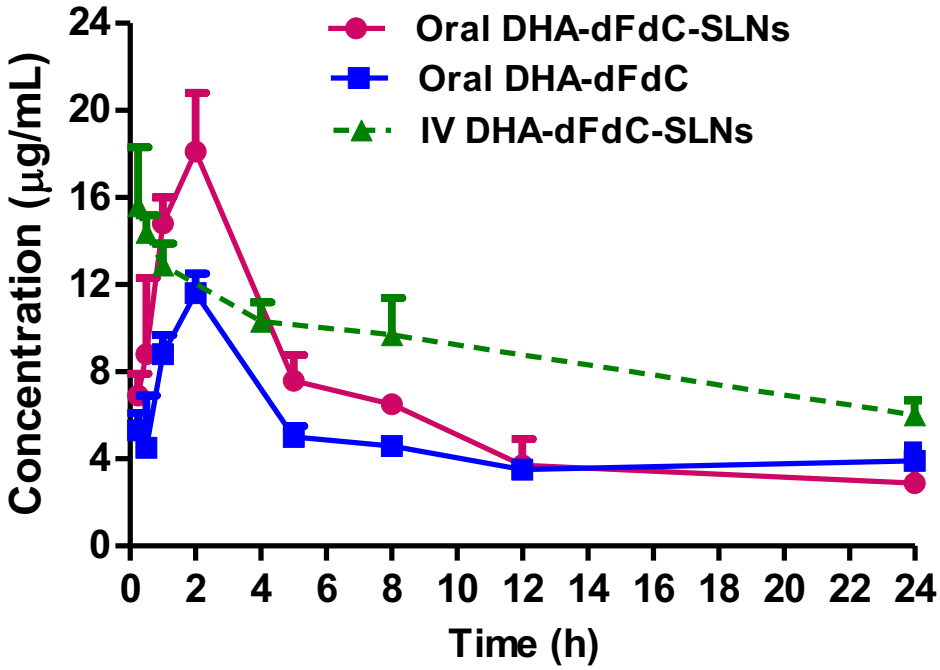


Fig.4.3

Plasma DHA-dFdC concentration-time curves after oral administration of DHA-dFdC-SLNs in suspension or DHA-dFdC in Tween 20-ethanol-water solution, or i.v. administration of DHA-dFdC-SLNs in suspension in healthy C57BL/6 mice. The dose of DHA-dFdC was 2 mg per mouse. Data are expressed as mean ± S.D. (n = 3)

Table 4.2 Selected pharmacokinetics parameters of DHA-dFdC in plasma followed by i.v. administration of DHA-dFdC-SLNs oral administration of DHA-dFdC in a Tween 80/ethanol/water solution or in DHA-dFdC-SLNs.

PK parameters	Oral administration		i.v. administration
	DHA-dFdC-SLNs	DHA-dFdC	DHA-dFdC-SLNs
Dose (mg)	2	2	2
k12 (1/h)	0.41	0.56	0.40
T 1/2 α (h)	1.10	1.07	0.53
T ½ β (h)	32.76	693147.18	25.58
T_{max} (h)	1.73	1.75	-
C_{max} (μg/mL)	17.01	10.50	-
AUC_{0-24} (μg*h/mL)	143.44	113.55	210.58
Fab%	**68.12**	-	-
Frel%	**126.32**	-	-

Abbreviations: AUC, total area under the plasma concentration-time curve form time zero to 24 h; C_{max}, peak plasma concentration; T_{max}, time to reach C_{max}; Frel %, relative oral bioavailability in percentage; Fab% absolute oral bioavailability in percentage

The plasma DHA-dFdC level after i.v. administration of DHF-dFdC-SLNs in healthy mice appeared to follow a two-compartment model with $AUC_{0\text{-}24h}$ value of 210.58 μg*h/mL. On the other hand, the plasma DHA-dFdC level in mice after oral administration of DHA-dFdC-SLNs followed an apparent adsorption phase and then a clearance phase, with a C_{max} of 17.01 μg/mL, T_{max} of 1.73 h, and $AUC_{0\text{-}24h}$ of 143.44 μg*h/mL. The absolute oral bioavailability of DHA-dFdC in the DHA-dFdC-SLNs was calculated to be 68.12% based on the $AUC_{0\text{-}24h}$ values shown in Table 4.2.

For a comparison, the plasma concentration of DHA-dFdC-time curve of the DHA-dFdC after it was oral administered in a Tween 80-ethanol-water solution is also showed in Fig. 4.3. The T_{max} was ~1.7 h, similar to that of the oral DHA-dFdC in SLNs (Table 4.2). However, the C_{max} and $AUC_{0\text{-}24h}$ values of the DHA-dFdC in solution were found to be 10.50 μg/mL and 113.55 μg*h/mL, respectively. Therefore, the bioavailability of DHA-dFdC in the DHA-dFdC-SLNs, relative to in the Tween 80-ethanol in water solution was 126.4%.

The exact mechanism by which the DHA-dFC in the DHA-dFdC-SLNs was absorbed into the blood circulation after oral gavage is unknown. In general, orally administered SLNs can be absorbed as intact particles through the microfold cells in the Peyer´s patches and then transported to the lymphatic system (22). However, others suggested that SLNs suffer from digestion or degradation in the GI tract, and only a very small fraction, if any, of orally administered SLNs can reach the blood circulation intact (23). Of course, DHA-dFdC can be released from the SLNs in the GI tract (Fig. 4.2), especially in the presence of lipases and co-lipases from pancreas, and then absorbed by passive diffusion or with the help of bile in the GI tract (24, 25).

As to the higher bioavailability of DHA-dFdC in SLNs relative to DHA-dFdC in Tween 80/ethanol/water solution, the DHA-dFdC in the solution may be susceptible to precipitation when orally administered, which can lead to a decrease in its bioavailability (6). It is also thought that the higher levels of exogenous lipids from SLNs after digestion (i.e. exogenous solubilizing components), relative to endogenous solubilizing components in the GI tract, may lead to a change in the nature of the GI fluid and enhance DHA-dFdC solubilization (25). Nonetheless, since the

solution of DHA-dFdC contains Tween 80, which may explain the relatively high oral bioavailability of DHA-dFdC in the solution (26). Tween 80 can be digested by intestinal cells to release oleic acid, which is used to increase the basolateral secretion of triglyceride-rich lipoproteins such as chylomicrons, increasing the lymphatic uptake of lipophilic drug (26). In addition, Tween 80 can inhibit intestinal P-gp efflux, increasing the concentration and residence time into the enterocyte of P-gp substrate (27). Although Tween 80 can inhibit intestinal P-gp activity, it is least effective than TPGS (28). It was reported that TGPS as emulsifier in a paclitaxel-polymeric nanoparticle formulation helped to increase the oral bioavailability of paclitaxel by 10-fold, as compared to oral Taxol (21). Furthermore, it was reported that TPGS1000-emulsified SLNs improved the intestinal absorption and relative oral bioavailability of docetaxel in rats (29). Therefore, the high oral bioavailability of DHA-dFdC in our DHA-dFdC-SLNs may be attributed in part to the presence of TPGS in the formulation as well.

4.4.4 Antitumor activity of DHA-dFdC-SLNs in a tumor-bearing mouse model

The antitumor activity of DHA-dFdC-SLNs was evaluated in a mouse melanoma model. Previously, we reported that DHA-dFdC-SLNs significantly inhibit the growth of B16-F10 tumor cells in culture and in mice when given intravenously. Consequently, we chose to use B16-F10 tumor-bearing mice to test the antitumor activity of DHA-dFdC-SLNs when given orally. DHA-dFdC-SLNs were orally gavaged at a dose of 250 μg of DHA-dFdC per mouse daily for a total of 12 days (with a two-day rest in the middle). As shown in Fig. 4.4, 50% of mice in the untreated group reached the endpoint on day 16. Oral DHA-dFdC-SLNs significantly improved the survival, as compared to the untreated group ($p < 0.05$). Oral DHA-dFdC in Tween 80/ethanol/water solution did not significantly affect mouse survival as compared to mice left untreated, which is surprising because the bioavailability of the DHA-dFdC in the Tween 80/ethanol/water solution was ~54% (Table 4.2). Toxicity associated with the DHA-dFdC in Tween 80/ethanol/water solution was likely related to the lack of survival advantage of the DHA-dFdC solution over

untreated mice, as 62.5% of the mice orally gavaged with the DHA-dFdC in Tween 80/ethanol/water solution showed signs of toxicity such as a body weight decrease of more than 20% (i.e. one mouse) or severe tumor ulceration (i.e. four mice). The exact reasons underlying the toxicity of the DHA-dFdC in the Tween 80/ethanol-water solution remains unknown, but should be related to the Tween 80-ethanol-water solution, although the amounts of Tween 80 and ethanol taken by mice from the DHA-dFdC in Tween 80/ethanol/water solution were within the normal range recommended for preclinical animal study (i.e. water with not over 10% Tween 80 and 5% solution of ethanol for one month is well tolerated) (30, 31). Nonetheless, the mouse survival data clearly indicate our SLN formulation also reduced the oral toxicity of DHA-dFdC.

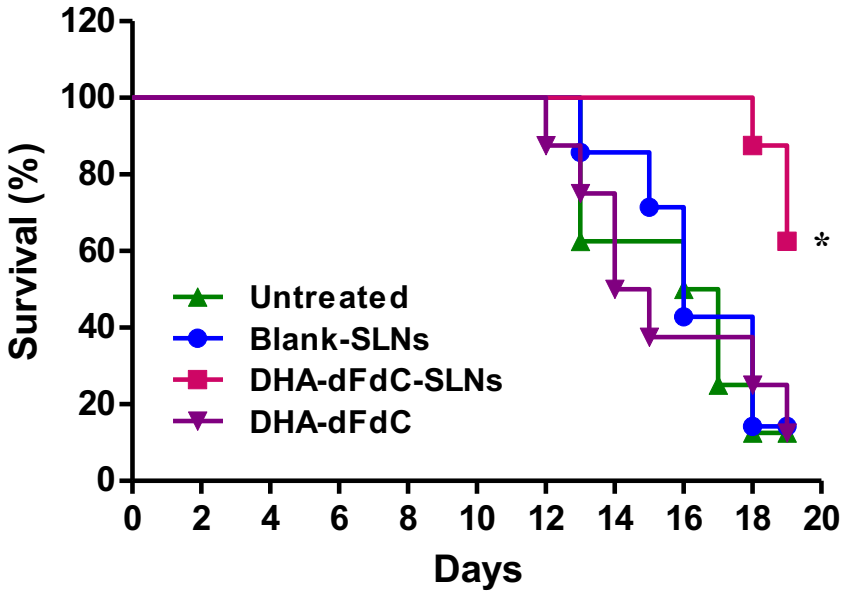


Fig. 4.4 Survival curves of B16-F10 tumor-bearing mice after oral treatment with DHA-dFdC-SLNs. Tumor cells were injected (s.c.) on day 0. On day 7, mice were randomized and orally gavaged with DHA-dFdC-SLNs in suspension or DHA-dFdC in a Tween 80-ethanol in water solution. As controls, mice received DHA-dFdC-free SLNs (blank-SLNs) or left untreated. * $p < 0.05$, DHA-dFdC-SLNs vs. all other groups (Log-rank Mantel-Cox test. Data shown are mean ± S.D. (n = 7-8).

4.5 CONCLUSION

In the present study, we reported that it is feasible to orally administer DHA-dFdC using DHA-dFdC-SLNs. The oral bioavailability of DHA-dFdC in the DHA-dFdC-SLNs was ~68%, and oral DHA-dFdC-SLNs improved the survival of tumor-bearing mice.

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Chapter 5: General Conclusion

DHA-dFdC is a novel compound synthesized by conjugating DHA and dFdC in the 4'-*N* position. DHA-dFdC has potent, broad spectrum antitumor activity. In this dissertation, we tested the toxicity of DHA-dFdC dissolved in a Tween 80-ethanol-water solution and developed a solid-lipid nanoparticle formulation of DHA-dFdC (i.e. DHA-dFdC-SLNs) that improves the water solubility and chemical stability of DHA-dFdC. In addition, this formulation modifies the plasma pharmacokinetic parameters of DHA-dFdC after intravenous or oral administration. Finally, DHA-dFdC-SLNs also improve the antitumor activity of DHA-dFdC after intravenous or oral administration.

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Vita

Solange Alondra Valdes Curiquen was born in Santiago, Chile in 1980. She received a Bachelor's degree of Biochemistry in 2009 from the University of Chile. She joined the Graduate School at the University of Texas at Austin in Fall 2013. During this time, she worked as graduate research assistant in Cui' Lab as well as teaching assistant at the college of Natural Science at the University of Texas at Austin.

Permanent e-mail: solvaldes@gmail.com

This dissertation was typed by the author.